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Interneuron diversity and function in the zebrafish locomotor circuits

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Interneuron diversity and function in the zebrafish locomotor circuits

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother

ABSTRACT

One of the most essential features of an animal's behavior is the ability to navigate and interact with its environment. The generation of the locomotor rhythm is carried out by networks of neurons in the spinal cord, so called central pattern generators (CPGs). Deficiencies in these neuronal networks can have detrimental effects on an animal's well-being and survival. The overall aim of this thesis is to understand how motor neurons and commissural V0 interneurons, influence the operation of the spinal locomotor CPGs.

Traditionally, the CPG networks in the spinal cord are thought to be composed of different interneuron classes with homogeneous properties, providing the output to the motor neurons, which are 'passive recipients' of the final motor program. In the first study of this thesis, we show that the motor neurons in adult zebrafish can influence the CPG output by retrograde signaling to the rhythm-generating V2a interneurons via gap junctions. This finding suggests that the motor neurons are an integral part of the CPG network instead of a passive component.

The two latter studies included in this thesis focus on one of the interneuron classes of the CPG, the V0 interneurons. This interneuron class comprises both excitatory (V0v) interneurons and inhibitory (V0d) interneurons. In the third study of this thesis, we show that the majority of glycinergic V0d interneurons in larval zebrafish are active only at fast frequency locomotion, and that they are homogenous also in terms of their morphologies. By contrast, previous studies in mice suggest that the V0d interneurons are responsible for left-right alternation during slow-frequency locomotion, whereas the V0v interneurons take over this task at high-frequencies. We further show, in this thesis, that the adult zebrafish V0v and V0d sub-populations are heterogeneous and comprise neurons which become active at slow, intermediate, or fast locomotor frequencies. Interestingly, the V0v interneurons had a predominance of fast neurons, whereas the V0d interneurons had a larger proportion of slow neurons. Hence, we show that both the V0v and the V0d sub-populations of the V0 class of interneurons in adult zebrafish are more diverse in their properties than has previously been assumed for the interneuron classes in the spinal cord. Furthermore, the V0d interneurons undergo changes to their morphological characteristics and their activity pattern during locomotion as the fish develops into adulthood.

Overall, the work comprising this thesis shows that one of the interneuron classes in the CPG networks, the V0 interneurons, is more diverse than has previously been shown; it can be sub-divided into groups of neurons active at different speeds of locomotion, thereby carrying out different functions from each other in the network. In addition, these interneurons are organized differently at earlier stages of development, indicating that the neurons undergo functional changes during the maturation of the networks. Furthermore, this work has shown that the motor neurons are not merely passive conveyers of upstream generated motor programs, but actively participate, and thus are an integrated component of the CPG networks.

LIST OF PUBLICATIONS

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CONTENTS

LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BMP	Bone morphogenetic protein
ChaT	Choline Acetyl Transferase
CiA	Circumferential ascending
CiD	Circumferential descending
CoBL	Commissural bifurcating longitudinal
CoLA	Commissural longitudinal ascending
CoLo	Commissural local
CoPA	Commissural primary ascending
CoSA	Commissural secondary ascending
CPG	Central pattern generator
CSF-c	Cerebrospinal fluid-contacting
DoLA	Dorsal longitudinal ascending
E-IN	Excitatory interneuron
FF	fast fatiguing
FR	fatigue resistant
GABA	Gamma-Aminobutyric acid
GFP	Green fluorescent protein
GlyT2	Glycine transporter 2
hsp	Heat shock protein
I-IN	Inhibitory interneuron
KA	Kolmer-Agduhr
LMC	Lateral motor column
m2	muscarinic 2
MCoD	Multipolar commissural descending
MMC	Medial motor column
MN	Motor neuron
MS	Multiple sclerosis
NpHR	*Natronomonas pharaonis* Halorhodopsin
RMP	Resting membrane potential
Shh	Sonic hedgehog
S-type	slow-twitch
Tg	Transgenic
UAS	Upstream activation sequence
UCoD	Unipolar commissural descending
V0-e	V0 excitatory
V0-i	V0 inhibitory
VeMe	Ventral medial
vGluT2	Vesicular glutamate transporter 2

1 INTRODUCTION

1.1 STUDYING LOCOMOTION

According to the encyclopedia Britannica, locomotion is defined as: ‘any of a variety of movements among animals that results in progression from one place to another’. Such progression is vital for animals to fulfill behavioral needs such as foraging, hunting, feeding, escaping, mating, and seeking shelter. Understanding the neural, synaptic and molecular mechanisms underlying these diverse and complex behaviors is essential because impairments of the locomotor circuit function is observed in a variety of traumatic and neural disorders including, among others, MS, ALS, Huntington’s disease Parkinson’s disease and spinal cord injury.

The study of the neural mechanisms underlying locomotion dates back to over a century ago. In 1906, Sherrington put forward the idea that the nervous system was not, as previously thought, composed of a single interlinked network. Using the cat as a model system, he further suggested that progression, or locomotion, was the result of a proprioceptor-mediated sequence of movements with reciprocal inhibition of antagonistic musculature, resulting in the alternation seen during stepping (Sherrington, 1906). In 1911, T. Graham Brown showed that proprioception is more likely to play a regulatory role, since removal of proprioceptive inputs does not lead to an inability to locomote. He instead proposed the idea that the networks generating locomotion are intrinsic, or central, to the spinal cord and consist of antagonistic half-centers (Brown, 1911). The work of Sherrington and Brown laid the foundations for the modern-day field of motor control.

Sherrington’s work on reflex interaction in the cat was continued by, among others, John Eccles and later Anders Lundberg. In their work, they emphasized that locomotion was a result of a series of reflex chains (reviewed in Stuart and Hultborn, 2008). This view was later thoroughly challenged in a review by Sten Grillner (Grillner, 1975). In the light of locomotion being evoked in deafferented monkeys, dogs and bony fish, Grillner wrote that: ‘it therefore can be concluded that rhythmic activity with maintained intersegmental coordination can be generated within the spinal cord without any phasic reflex input’ (Grillner, 1975). Grillner concluded by emphasizing his hope that the argument for either a peripheral or central control of locomotion would be buried and replaced by the consideration that both systems could be important for the generation and maintenance of appropriate locomotor output (Grillner, 1975).

1.2 CENTRAL PATTERN GENERATORS

The discovery that locomotion in vertebrates is centrally controlled in species as diverse as fish and monkeys, led to studies of the neural basis of locomotion being carried out in various animal models. Vertebrates such as the lamprey and the *Xenopus laevis* tadpole were accessible enough to allow for detailed investigation of the neuronal networks underlying locomotion by anatomical approaches, as well as electrophysiological single and paired cell recordings from spinal motor neurons and interneurons during fictive locomotor activity (Roberts, Soffe and Clarke, 1983; Dale and Roberts, 1984, 1985; Wallén *et al.*, 1985; Dale, 1985; Dale and Grillner, 1986; Dale *et al.*, 1986; Moore, Hill and Grillner, 1987; Buchanan *et al.*, 1987, 1989; Buchanan and Grillner, 1988; Buchanan and Grillner, 1987; Grillner and Matsushima, 1991; Buchanan, 1996; Li *et al.*, 2001; Li, 2004; Zhang *et al.*, 2009). These studies led to a detailed scheme of the spinal locomotor networks, or central pattern generators (CPGs). However, it ought to be mentioned that the idea of a CPG also derived from work carried out in invertebrates, specifically on the flight control system of locusts, the lobster stomatogastric ganglion, and the neural circuits controlling the swimmerets in crayfish (Hughes and Wiersma, 1960; Wilson and Wyman, 1965; Selverston, a i, Miller, 1980; Eisen, JS, 1982; Miller and Selverston, 1982a, 1982b, 1982b; Mulloney, 2010).

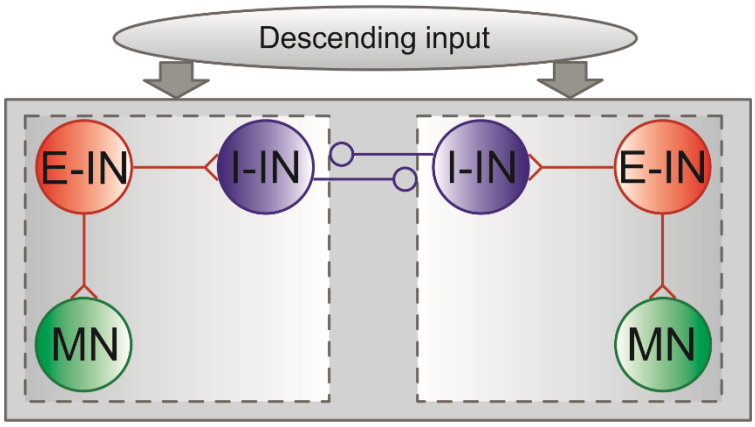

Figure 1. Basic organization of the vertebrate CPG. Excitatory interneurons (E-IN) on either side of the spinal cord provide excitation to the motor neurons (MN) and commissural inhibitory interneurons (I-IN). The crossing inhibition ensures left-right alternation between the two sides of the spinal cord. Adapted from (Goulding, 2009; Grillner and Jessell, 2009).

In the vertebrate locomotor networks, the motor neurons on either side of the body receive input from the upstream located CPGs, consisting of a range of interneuron classes. The motor neurons have traditionally not been viewed as part of the CPG due to the 'output' nature of their signal transduction (Grillner and Wallén, 1985). Ipsilaterally projecting excitatory interneurons provide excitation to the downstream motor neurons and to other excitatory and inhibitory interneurons in the same hemi-cord. In addition, contralaterally projecting inhibitory interneurons project to the excitatory interneurons and motor neurons located on the opposite side and inhibit them to ensure left right alternation of activity between the two sides (Fig. 1) (Butt and Kiehn, 2003; Grillner, 2003; Brownstone and Wilson, 2008; Goulding, 2009; Kiehn, 2016). The CPGs can maintain locomotion without continuous descending or proprioceptive input (Grillner and Zangger, 1975; Delcomyn,

1980; Alstermark *et al.*, 1981; Jankowska and Lundberg, 1981; Pearson, 1993; Rossignol, 2006). This organization of the spinal neuronal networks, along with observational studies of the control of stepping in various vertebrates, appeared to suggest that there is a common, conserved organization of the circuitry underlying locomotion across species.

1.3 COMMON TRANSCRIPTION CODE ACROSS VERTEBRATE SPECIES

To thoroughly investigate the role that different neuronal populations play in the locomotor circuitry, it is beneficial to be able to make targeted recordings or manipulations of neurons belonging to the same class. The sequencing of the genome of different animals made it possible to target neurons expressing a specific set of transcription factors through the use of transgenic animals. Furthermore, it has validated the use of diverse model organisms to study the spinal neuronal circuits, since the transcription factor code in the developing spinal cord has been found to be preserved throughout the vertebrate lineage (Kiehn and Kullander, 2004; Goulding, 2009; Grillner and Jessell, 2009).

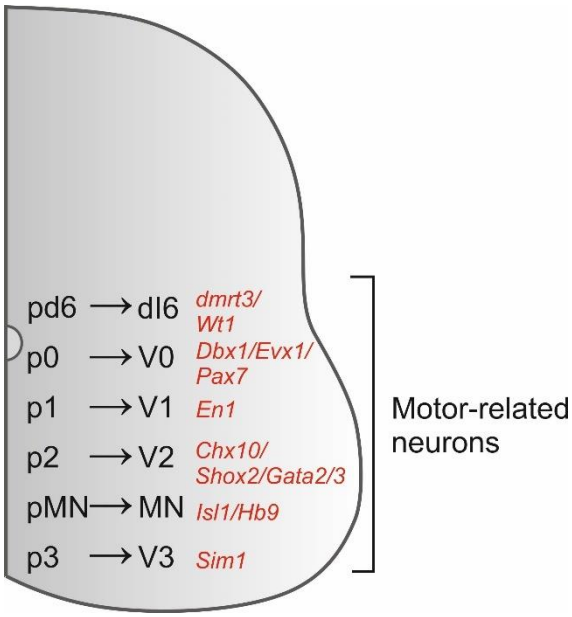

Figure 2. The motor function-related neuronal classes in the mammalian spinal cord. There are five ventral progenitor domains which give rise to four motor-related interneuron classes (V0-V3) and the motor neurons (MN) in the spinal cord. One dorsal progenitor domain (dI6) gives rise to a dorsal interneuron class also involved in locomotor output. Adapted from (Goulding, 2009; Alaynick, Jessell and Pfaff, 2011).

Across vertebrate species, the developing spinal cord contains five ventral progenitor domains, defined by their combined expression of a set of homeodomain transcription factor proteins which are differentially regulated by a dorso-ventral gradient of bone morphogenetic proteins (BMP) and sonic hedgehog (Shh). These five progenitor domains give rise to four different classes of CPG interneurons (V0-V3) and one class of motor neurons (Fig. 2) (Ericson *et al.*, 1997; Briscoe *et al.*, 2000; Jessell, 2000; Liem, Jessell and Briscoe, 2000; Lee and Pfaff, 2001; Goulding *et al.*, 2002; Goulding, 2009; Arber, 2012). In the dorsal spinal cord there are six progenitor domains that give rise to somatosensory relay neurons (dI1-dI3),

and association interneurons (dI4-dI5), as well as one class of CPG interneuron (dI6) which express *Lbx1*(Jessell, 2000; Lee and Pfaff, 2001; Goulding *et al.*, 2002; Andersson *et al.*, 2012; Dyck, Lanuza and Gosgnach, 2012; Vallstedt and Kullander, 2013). Although the dorsal interneurons are mainly responsible for sensory input and not considered part of the CPG as such, some of them, such as the dI3 interneurons, make direct contact with motor neurons and mediate cutaneous input important for grasping (Bui *et al.*, 2013).

The advent of new techniques has made it possible to genetically target the different neuron populations in the CPG of the spinal cord to investigate their differentiation, morphology and functional roles during locomotion.

1.4 CPG NEURON CLASSES IN MICE

1.4.1 V0 interneurons

In the mouse, the V0 interneurons express the transcription factors dbx1 and dbx2 and have four different transmitter phenotypes: glutamatergic, glycinergic, GABAergic and cholinergic (Pierani *et al.*, 2001; Lanuza *et al.*, 2004; Goulding, 2009; Zagoraiou *et al.*, 2009). They can be divided into four groups: V0 dorsal (V0d) and V0 ventral (V0v), named so after their relative positions in the developing spinal cord, and V0 cholinergic (V0c) and V0 glutamatergic (V0g). The V0d population expresses Pax7 and are commissural inhibitory, mixed glycinergic/GABAergic whereas the V0v express Evx1 and Evx2 and are commissural glutamatergic interneurons (Pierani *et al.*, 1999, 2001; Moran-Rivard *et al.*, 2001; Lanuza *et al.*, 2004). Inhibitory V0d interneurons form inhibitory connections directly with motor neurons, whereas excitatory V0v interneurons likely make contact with local ipsilateral inhibitory interneurons on the other side of the cord, which in their turn inhibit the motor neurons (Kjaerulff and Kiehn, 1997; Butt and Kiehn, 2003; Lanuza *et al.*, 2004; Quinlan and Kiehn, 2007). This connectivity between commissural interneurons and motor neurons has also been found in the lamprey and the tadpole (Buchanan, 1982, 1999; Dale, 1985). Conversely, the V0c and V0g interneurons are ipsilateral and express the transcription factor Pitx2 and are transiently Evx1-positive, suggesting that they are a subgroup of V0v interneurons. The V0c interneurons appear to increase motor neuron excitability by reducing the afterhyperpolarization amplitude by acting on muscarinic m2 receptors, whereas less is known about the V0g interneurons (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009).

Deletion of dbx1, and the resulting loss of V0 interneurons in the mouse causes co-activation of the left and right ventral roots during locomotion, however, deletion of Evx1 positive neurons has no such apparent effect (Lanuza *et al.*, 2004). A later study has shown that deletion of Dbx1 positive neurons, i.e. all V0 interneurons, leads to a hopping gait, whereas deletion of Evx1 positive neurons, i.e. the V0v population causes hopping only at higher speeds of locomotion. Conversely, deletion of Pax7 positive neurons, i.e. the V0d population leads to a hopping gait only at low frequency locomotion (Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015). In accordance with some of these results, blocking inhibition in the spinal

cord has been reported to induce rhythmic, synchronous activity in rat, lamprey and zebrafish (Cohen and Harris-Warrick, 1984; McPherson, Buchanan and Kasicki, 1994; Cowley and Schmidt, 1995; Bracci, Ballerini and Nistri, 1996; E. Kremer, 1997; Kjaerulff and Kiehn, 1997; McDearmid, 2005; Gabriel *et al.*, 2008; Kyriakatos *et al.*, 2011).

The increase of endogenous release of acetylcholine increases the locomotor output in the isolated spinal cord preparation without affecting rhythmicity, hinting at the contribution of the V0c interneurons to the locomotor output (Miles *et al.*, 2007). In addition, genetic deletion of choline acetyl transferase (ChAT) leads to impairments in increasing motor neuron output and consequently modulating muscle force in a task-dependent manner (Zagoraiou *et al.*, 2009).

If the dbx1 gene is inactivated in mouse, the generation of V0 interneurons is impeded and instead neurons with a phenotype like that of the V1 class of interneurons are generated (Moran-Rivard *et al.*, 2001; Pierani *et al.*, 2001).

1.4.2 V1 interneurons

The V1 interneurons express the transcriptions factor Dbx2 but not Dbx1, along with En1. They are ipsilateral, inhibitory glycinergic/GABAergic and form monosynaptic connections with motor neurons (Pierani *et al.*, 1999; Saueressig, Burrill and Goulding, 1999; Wenner, O'Donovan and Matise, 2000; Moran-Rivard *et al.*, 2001). More recent work has shown that they are a diverse class of interneurons with differential expression of as many as 19 different transcription factors, indicating that there are different subtypes of V1 interneurons with distinct functions (Bikoff *et al.*, 2016). Renshaw cells and 1a inhibitory interneurons belong to the V1 class (Sapir, 2004; Alvarez *et al.*, 2005; Benito-Gonzalez and Alvarez, 2012). Ablation of the V1 interneurons leads to a slower step-cycle duration and a lengthening in the locomotor burst duration, suggesting that the V1 population is involved in regulating the speed of locomotion through burst termination (Gosgnach *et al.*, 2006; Nishimaru, 2006).

1.4.3 V2 interneurons

Dorsal to the progenitor domain that gives rise to the V1 interneurons is the p2 progenitor domain, from which V2 interneurons are generated. The V2 interneuron class can be subdivided into the glutamatergic V2a population, which express the transcription factors Lhx3 and Chx10, and the GABAergic V2b populations, which express Gata2 and Gata3 (Li *et al.*, 2005; Al-Mosawie, Wilson and Brownstone, 2007; Lundfald *et al.*, 2007). The inhibitory V2b interneurons are intermingled with the V1 population in the spinal cord, and their generation is dependent on Notch signaling. Indeed, when Notch signaling is suppressed, there is a decrease in V2b interneurons and an increased generation of V2a interneurons (Del Barrio *et al.*, 2007; Peng *et al.*, 2007; Goulding, 2009). In addition, a third type of V2 interneuron, the V2c has been shown to depend on the expression of Sox1, without which they become re-programmed into V2b interneurons. The function of the V2c interneurons remains to be determined (Panayi *et al.*, 2010).

In mice, the V2a interneurons have been proposed to play a role in left-right alternation through their connections with commissural interneurons (Crone *et al.*, 2008). Genetic ablation of V2a interneurons does not abolish locomotion but leads to a failure of left-right alternation at intermediate-to-fast speeds of locomotion, suggesting that this connectivity is speed-specific (Crone *et al.*, 2009). Furthermore, the V2a interneurons are heterogeneous in their firing patterns as well as their morphologies (Dougherty and Kiehn, 2010; Zhong *et al.*, 2010) and their recruitment appears to be linked to locomotor frequency, with an increase in frequency leading to an increased synaptic drive to the V2a interneurons (Zhong *et al.*, 2010; Zhong *et al.*, 2011). In addition, a subpopulation of the V2a interneurons express the transcription factor Shox2, indicated by a partial overlap of neurons expressing Chx10 and Shox2. When Shox2- expressing V2a interneurons are ablated, only a modest effect can be seen in the locomotor rhythm, however, deletion of vGluT2 from the entire Shox2-expressing population leads to a decrease in the locomotor frequency with no effect on left-right alternation. This suggests that the Shox2-positive V2a population could be involved in stabilizing the locomotor frequency, whereas the Shox2-only population is involved in the generation of the locomotor rhythm (Dougherty *et al.*, 2013).

Another population of neurons which has been suggested as the rhythm-generating entity is a subgroup of interneurons expressing the homeodomain transcription factor Hb9, which is also expressed by motor neurons. These interneurons are glutamatergic and ipsilaterally projecting and are rhythmically active during locomotion (Wilson, 2005; Kiehn, 2006; Brownstone and Wilson, 2008; Ziskind-Conhaim and Hinckley, 2008; Ziskind-Conhaim, L, Mentis, G., Wiesner, E P., Titus, 2010; Caldeira *et al.*, 2017). However, the rhythm-generating abilities of the Hb9 interneurons have been questioned due to unreliable activity on every locomotor cycle and a delay in the onset of their activity in relation to the activity in the ipsilateral ventral root (Kwan *et al.*, 2009). A recent study has shown that the Hb9 interneurons do not overlap with the non-V2a Shox2-expressing interneurons and suggests that these two populations could be responsible for rhythm-generation together (Caldeira *et al.*, 2017).

The inhibitory V2b interneurons have been shown to include some 1a inhibitory interneurons, indicating that these arise from two separate progenitor domains, p1 and p2 (Zhang *et al.*, 2014). Abolishing synaptic transmission in the V2b interneurons leads to some impairment of flexor-extensor coordination, however in order to fully eliminate it, a simultaneous abrogation of synaptic transmission also of the V1 interneurons is necessary. This indicates that V2b interneurons act together with the V1 interneurons to coordinate flexor-extensor alternation (Zhang *et al.*, 2014). More specific deletion of the V2b and V1 interneurons respectively by means of diphtheria toxin has shown that deletion of V2b interneurons leads to hyperextension of the hindlimb, whereas deletion of the V1 interneurons leads to hyperflexion of the hindlimb. These results suggest that the V2b and V1 interneurons are important for appropriate inhibition of the motor neurons innervating the extensor and flexor musculature respectively (Britz *et al.*, 2015).

1.4.4 V3 interneurons

The p3 progenitor domain gives rise to a set of Sim1-expressing glutamatergic interneurons (Briscoe *et al.*, 1999; Goulding *et al.*, 2002). These V3 interneurons are mainly commissural, however a small subset has ipsilateral axons, indicating that this neuronal class contacts both sides of the cord to carry out their function. Furthermore, the V3 interneurons make direct contact with motor neurons and CPG-related interneurons, including Renshaw and 1a inhibitory interneurons. When synaptic transmission is inhibited in the V3 interneurons, the locomotor rhythm becomes less stable, indicating that this neuron class is involved in producing a robust locomotor pattern, as well as balancing the activity between the two sides of the spinal cord (Zhang *et al.*, 2008).

1.4.5 DI6 interneurons

Although di6 interneurons derive from a dorsal progenitor domain, this population settles in the ventral spinal cord and has been postulated to be part of the locomotor CPG. These interneurons transiently express Lbx1 and/or Wt1 and Dmrt3 and have been shown to be composed of a mixed population of ipsilateral and commissural interneurons which make monosynaptic contact with motor neurons (Goulding *et al.*, 2002; Gross, Dottori and Goulding, 2002; Lanuza *et al.*, 2004; Goulding, 2009; Andersson *et al.*, 2012; Griener *et al.*, 2017). These interneurons can be divided into two groups based on their pattern of activity during locomotion, either tightly or loosely firing with the rhythm of the locomotor output, suggesting that they play more than one role during locomotion (Dyck, Lanuza and Gosgnach, 2012; Griener *et al.*, 2017). Moreover, deletion of the transcription factor Dmrt3, which is expressed in a subset of dI6 neurons, leads to a decrease in the number of commissural interneurons and an increased burst and inter-burst duration, irregular rhythm and difficulty in locomoting at higher frequencies (Andersson *et al.*, 2012).

1.4.6 Motor Neurons

The most ventral neuron class generated in the spinal cord is the motor neuron class. LIM homeodomain transcription factors are transiently expressed in motor neurons during development and the combinatorial expression of these gives rise to a columnar organization of the motor neurons in the spinal cord. The lateral motor column (LMC) contains neurons innervating the limb musculature and the medial motor column (MMC) neurons innervating axial musculature. Furthermore, the generation of all motor neurons is dependent on the LIM-homeodomain transcription factor Isl1, and deletion of Isl1 impairs motor neuron generation (Pfaff *et al.*, 1996; Jessell, 2000). Mammalian motor neurons are named after the muscle fiber type they innervate and the motor neuron and muscle fiber are described as a ‘motor unit’. There are three types of motor units. Slow twitch (S-type), fast fatiguing (FF-type) and fast fatigue resistant (FR-type). The terminology refers to the force of the output from the different units. The three different muscle fiber types are intermingled in the musculature and are hence difficult to target specifically (Heckman and Enoka, 2012). Interestingly, impairment of motor neuron generation appears to affect proper generation of the En1-expressing V1 interneurons, indicating that the motor neurons provide further signaling

molecules to guide proper interneuron generation (Pfaff *et al.*, 1996). In fact, it has been found that early born motor neurons at the limb levels of the spinal cord guide later born motor neurons by means of retinoid signaling, suggesting that retinoid signaling from the early born motor neurons could be important for the generation of later born motor neurons and perhaps also some interneurons (Sockanathan and Jessell, 1998). Ephrins have also been shown to be involved in appropriate signaling during axonal pathfinding in limb motor neurons (Helmbacher *et al.*, 2000; Jessell, 2000).

The motor neurons have long been considered passive recipients of signals from the upstream CPG networks of interneurons, however they have been shown to possess some modulatory roles as well. For example, it has been shown in mammals that the motor neurons have collaterals that make contact with Renshaw cells (Eccles, Fatt and Koketsu, 1954; Eccles, J. C., Eccles, R. M., Iggo, A, Ito, 1961; Hultborn H, Lindström S, 1979) and that the signaling is mixed glutamatergic and cholinergic. Interestingly, the two signaling mechanisms appear to be segregated from each other (Nishimaru *et al.*, 2005; Lamotte d'Incamps *et al.*, 2017). Furthermore, recent work has shown that motor neurons in mice send axon collaterals to other motor neurons. These connections are stronger between motor neurons of the fast type and are purely glutamatergic. This kind of connectivity could work to sequentially recruit motor units in motor tasks of increasing force, or to amplify a motor response (Bhumbra and Beato, 2018). Optogenetic inactivation of motor neurons by inactivation of Isl1-positive neurons decreases the frequency and perturbs the phasing of the locomotor rhythm, partially via AMPA-mediated signaling. These effects have been suggested to be due to a non-specific excitation of the CPG by transmitter release from the motor neuron dendrites or somata, or mediated via an undiscovered excitatory interneuron class, rather than effects of motor neuron collaterals (Falgairolle *et al.*, 2017).

Influencing signaling from motor neurons to the upstream CPG has also been reported in leech crawling (Rotstein, Schneider and Szczupak, 2017). Hence, it is becoming apparent that motor neurons have more sophisticated roles in locomotion than has been previously considered.

1.5 THE ZEBRAFISH AS A MODEL SYSTEM TO STUDY LOCOMOTION

The studies in mammals using a transcription factor-based approach have provided important insight into how the different classes of spinal neurons might be active during locomotion, and what their specific contributions towards the locomotor output might be. However, due to constraints of the animal models used, these classes have largely been treated as homogenous entities where all the neurons belonging to the same class are presumed to carry out the same function in the network. As the study of the V1 population in mouse by Bikoff et al shows, the picture might be more complex than this (Bikoff *et al.*, 2016). The zebrafish as a model system to study locomotion has the advantage of being amenable to genetic manipulations, as well as being accessible enough to perform intracellular recordings from

one, or multiple neurons, during evoked locomotion in the intact spinal network. This allows for a thorough mapping of function at the single-cell level and can consequently discriminate any differential contribution by different neurons of the same population to the final locomotor output. Furthermore, the zebrafish can be studied at different stages of development using the same, or very similar techniques, making it an excellent model to investigate the same class of neurons over the course of development.

The spinal neurons in the zebrafish have initially been studied in the larva (3-6 dpf) and were described and characterised on the basis of their morphological characteristics and neurotransmitter phenotype (Bernhardt *et al.*, 1990; Hale, Ritter and Fetcho, 2001; Higashijima, Mandel and Fetcho, 2004; Higashijima, Schaefer and Fetcho, 2004). The glutamatergic neurons described were the multipolar commissural descending (MCoD), the unipolar commissural descending (UCoD), the circumferential descending (CiD), the commissural primary ascending (CoPA) and the commissural secondary ascending (CoSA), whereas the glycinergic neurons were identified as the commissural bifurcating longitudinal (CoBL), the commissural longitudinal ascending (CoLA), the circumferential ascending (CiA), the commissural local (CoLo), as well as some CoSAs (Bernhardt *et al.*, 1990; Hale, Ritter and Fetcho, 2001; Higashijima, Mandel and Fetcho, 2004; Higashijima, Schaefer and Fetcho, 2004; Liao and Fetcho, 2008; Satou *et al.*, 2009). In addition, GABAergic neurons were identified in the larval zebrafish. These included the Kolmer-Agduhr cells (KA), also known as cerebrospinal fluid-contacting neurons (CSF-c), and the dorsal longitudinal ascending (DoLA) (Bernhardt RR, Patel CK, Wilson SW, 1992; Higashijima, Schaefer and Fetcho, 2004). While this terminology is still retained for describing the larval neurons, more recent studies have focused on using the transcription factor approach when studying the spinal populations, making the findings more relatable to similar work in other vertebrates.

1.5.1 V0 interneurons

In the larval zebrafish, the V0 interneurons express the transcription factor dbx1b. Like in the mouse, they can be divided into excitatory glutamatergic (V0-e), expressing Evx2, and inhibitory glycinergic or GABAergic (V0-i), expressing Pax2. The V0-e interneurons display variable morphologies, with neurons having a commissural axon that is ascending, descending or bifurcating. The ascending V0-e interneurons are born first, followed by the bifurcating and lastly the descending. The previously mentioned MCoD and UCoD neurons have been shown to be part of the descending V0-e interneurons (Satou, Kimura and Higashijima, 2012). No studies have yet looked at the activity of the V0-e interneurons in larval zebrafish, however previous work has shown that MCoD interneurons are active mainly at low frequencies of locomotion and switch off when the frequency increases (McLean *et al.*, 2007). In support of a role in slow locomotion, ablating the MCoDs impairs slow frequency swimming (McLean *et al.*, 2007). The activity of the V0-e interneurons has been further studied in the adult zebrafish and the results from that study are part of this thesis (Björnfors and El Manira, 2016).

The inhibitory V0-i interneurons have more homogenous morphologies. V0-i interneurons are almost exclusively commissural bifurcating, with the descending axonal process extending further than the ascending process (Satou, Kimura and Higashijima, 2012). The GABAergic neurons are commissural bifurcating, but the axon remains local and, in addition, they have ipsilateral axonal branches. The activity of the V0-i interneurons has not been studied; however, the morphology of the previously described glycinergic CoBL interneuron is similar to that defined for the V0-i glycinergic interneurons (Satou, Kimura and Higashijima, 2012). CoBL interneurons have been shown to be active during locomotor behaviors such as swimming, struggling and escape, however, it is not known if they are generated from the V0 domain or from the dI6 domain which is just dorsal to the V0 domain (Liao and Fetcho, 2008; Goulding, 2009). Work published in abstract form has suggested that the glycinergic V0-i interneurons are exclusively recruited at high frequencies, whereas Dmrt3-expressing dI6 interneurons are recruited at slow to intermediate frequencies (Kishore *et al.*, 2015). Work included in this thesis aims to clarify the activity pattern of the larval glycinergic V0-i interneurons, as well as characterizing these interneurons in the adult zebrafish.

Although no cholinergic V0 interneurons have directly been described in the spinal cord of zebrafish, a recent study has found that activation of muscarinic receptors appears to alter the excitability of motor neurons by potentiating neurons active at slow and intermediate speeds, while suppressing those active at faster speeds (Bertuzzi and Ampatzis, 2018). Whether these neurons are equivalent to the mouse V0c interneurons remains to be seen.

1.5.2 V1 interneurons

The V1 interneurons in the spinal cord of zebrafish express En1, like the mouse, or more specifically its homologue Eng1b. These neurons may also express dbx1a/1b/2, however the division between neurons expressing the different dbx genes are more diffuse in zebrafish compared to the mouse (Gribble, Nikolaus and Dorsky, 2007). A study of the En1- expressing neurons in larval zebrafish has shown that they correspond to the CiA interneurons. They are inhibitory glycinergic and project ipsilaterally. Using paired recordings, their targets have been determined to include both the CoPA interneurons as well as ventral motor neurons (Higashijima, 2004). The CoPA neurons relay sensory input from Rohon-Beard (RB) neurons. This suggests that the En1- positive neurons perform a dual function; shaping the motor pattern and providing sensory gating (Higashijima, 2004). This dual function has also been described for the En1-positive interneurons in Xenopus tadpoles (Sillar and Roberts, 1988; Li, Soffe and Roberts, 2002; Li, 2004). The V1 interneurons have not been studied yet in the adult zebrafish.

1.5.3 V2 interneurons

The V2 interneurons, specifically the V2a population, have been more extensively studied in both the larval and adult zebrafish than the V0 and V1 interneurons. Different levels of Notch signaling gives rise to an asymmetric division of p2 cells into V2a/V2b pairs. Zebrafish V2a

interneurons express the Chx10 homologue Alx, whereas V2b interneurons express Scl (Kimura, Satou and Higashijima, 2008). The Alx expressing V2a interneurons include the previously characterized CiD interneurons. They are glutamatergic and make up the large majority of ipsilaterally projecting excitatory interneurons in the zebrafish spinal cord (Kimura, 2006). In larval zebrafish, the V2a interneurons have been shown to be both necessary and sufficient to initiate locomotion in the spinal cord. Ablation of V2a interneurons in the mid-body region of the spinal cord leads to an increased threshold for initiation of locomotion but has no effect on the left-right alternation (Eklof-Ljunggren *et al.*, 2012). Similarly, optogenetic activation of the V2a interneurons can initiate locomotion (Ljunggren *et al.*, 2014). Hence, there appears to be a difference in the contribution of the V2a interneurons to locomotion in zebrafish compared to mice, where they are suggested to play a role in left-right alternation by contacting commissural interneurons (Crone *et al.*, 2008). The Hb9 interneurons proposed to be involved in rhythm-generation in mouse have been identified in the adult zebrafish, although their activity has not yet been determined (Stil and Drapeau, 2016).

Based on their activity during electrophysiological recordings, embryonic V2a interneurons can be divided into neurons active only during strong movements such as high frequency swimming and escape, and neurons active during slow movements, such as slow swimming. Strong movement V2a interneurons are born first and located dorsally, whereas slow movement V2a interneurons are later born and occupy a more ventral position in the cord (Kimura, 2006). This pattern of timing corresponds to the timing of the appearance of fast, vigorous behaviors versus more fine-tuned movements (McLean and Fetcho, 2009). In addition, electrical and chemical connections between V2a interneurons have been found for both slow and fast movement V2a interneurons (Kimura, 2006).

Studies of the V2a interneurons in larval zebrafish have shown that a large proportion are active during faster frequencies of locomotion whereas it is the MCoD interneurons that are active during slow frequency swimming (McLean *et al.*, 2007). Furthermore, within the V2a interneuron population, different sets of V2a interneurons are active at lower versus higher frequencies of swimming, with the lower frequency-activated neurons becoming inactive at higher frequencies (McLean *et al.*, 2008). In addition, the morphology of larval V2a interneurons differs between the dorsal, early born and the ventral, later born neurons in that the more dorsal V2a interneurons have more extensive descending projections than their ventral counterparts (Menelaou, Vandunk and McLean, 2014). It has been suggested that the larval V2a interneurons are segregated in two separate ‘streams’ providing excitation dorsally and ventrally to the primary motor neurons which in their turn innervate dorsal or ventral musculature. This mechanism possibly enables a more fine-tuned postural control (Bagnall and McLean, 2014).

In adult zebrafish, the V2a interneurons can be divided into three groups based on their activity pattern during locomotion: slow, intermediate and fast. The slow V2a interneurons are active across all frequencies of locomotion. The intermediate V2a interneurons reach their

recruitment threshold for firing action potentials at intermediate frequencies of locomotion, however, once activated they remain active at frequencies above the recruitment frequency. The fast V2a interneurons follow the same pattern as the intermediate but have a recruitment threshold at high frequencies and are hence only active at the fastest frequencies of locomotion (Ausborn, Mahmood and El Manira, 2012; Ampatzis *et al.*, 2014).

This pattern of recruitment corresponds to what has been found for the motor neurons in adult zebrafish, which in turn correlates with the muscle fiber type that the motor neurons innervate: slow, intermediate or fast (Gabriel *et al.*, 2011; Ampatzis *et al.*, 2013). Just like in embryonic zebrafish, the adult zebrafish V2a interneurons make both electrical and synaptic connections with motor neurons. Interestingly, these connections are strongest between neurons belonging to the same group - slow, intermediate or fast - and weaker between neurons of adjacent groups, i.e. slow-to-intermediate or intermediate-to-fast. Fast and slow neurons are never connected (Ampatzis et al., 2014).

1.5.4 V3 interneurons

The V3 interneurons in zebrafish have not been directly identified. However, the ventral medial (VeMe) interneurons have been suggested to belong to the V3 class since they have a similar morphology, and glutamatergic transmitter phenotype, as V3 interneurons in the mouse (Hale, Ritter and Fetcho, 2001; Higashijima, Mandel and Fetcho, 2004; Higashijima, Schaefer and Fetcho, 2004; Goulding, 2009). The UCoD interneurons were also previously thought to be V3 interneurons based on the same criteria (Goulding, 2009), however, they were later shown to arise from the V0 domain (Satou, Kimura and Higashijima, 2012).

1.5.5 Motor neurons

In the zebrafish, the motor neurons innervating the axial musculature can be largely divided into early-born primary, and later-born secondary subtypes. In adult zebrafish, there are three primary motor neurons per hemi-segment and they each have a specific pattern of innervation (Myers, 1985; Westerfield, McMurray and Eisen, 1986; Liu and Westerfield, 1988). The primary motor neurons are mainly active during the most forceful movements such as fast swimming, struggling and escape, whereas the secondary motor neurons, which are considerably higher in number, are active during regular swimming of slow and intermediate frequencies (Liu and Westerfield, 1988). The secondary motor neurons can be further divided into slow, intermediate and fast according to which muscle fiber type they innervate. This grouping also corresponds to the frequency of locomotion at which they reach firing threshold as described above. Furthermore, the motor neurons in the adult zebrafish follow a topographical organization, with the slow motor neurons occupying a more ventral and lateral position; the intermediate a slightly more dorsal and medial position, and the fast motor neurons occupying the most dorso-medial position (Gabriel *et al.*, 2011; Ampatzis *et al.*, 2013). Such a topographic organization is also found in the larval zebrafish motor neurons (McLean *et al.*, 2007).

In accordance with reports in mammals, larval zebrafish primary motor neurons appear to have axon collaterals which seem to project selectively dorsal or ventral in the spinal cord depending on the primary's soma location. Although the actual targets of these collaterals have not been identified, it has been suggested that the collaterals could provide re-enforcement to the topographical recruitment of spinal neurons. In addition, the motor neurons in adult zebrafish have been shown to have an influence on the locomotor network via gap junctions. The findings of this study make up part of this thesis (Song *et al.*, 2016).

1.6 SUMMARY

Taken together, the studies and their results described above comprise only a fraction of the work that has been carried out in the pursuit of understanding the organization of the spinal locomotor CPGs. Already over 60 years ago there was a sense that there was a certain degree of similarity between the nervous systems of different vertebrates. Many different animal models, from the cat to the lamprey, were being, and are still being, utilized to study the underlying principles of movement. The advent of a transcription factor approach has further validated cross-species studies and comparisons between different systems. Described above are some of the similarities and differences between two animal models: the mouse and the zebrafish. A remarkable number of findings appear to be the same in both species, with a few deviations. This illustrates that many aspects of the locomotor circuitry are conserved among the vertebrate species, from smaller networks consisting of fewer neurons, to more complex circuits involving additional neuron types. An interesting point is the comparison between the larval and the adult zebrafish nervous systems. Although largely the same, some differences in both organization and function seem to exist, indicating that the CPGs are plastic and vary not only between species but also within the same species, albeit executing the same overall goal which is locomotor movements.

2 AIMS

The overall aim of this thesis is to determine whether there is greater diversity of function within the different classes of interneurons and motor neurons than has previously been thought. Specifically, the following aims will be addressed:

- To determine whether the spinal motor neurons are ‘passive conveyers’ or whether they are an integrated part of the CPGs.

- To investigate the functional organization of the V0v sub-class of V0 interneurons in the adult zebrafish spinal cord.

- To find out how the glycinergic V0d sub-class of V0 interneurons is organized in the larval and adult zebrafish and elucidate the contribution of this neuron class to the locomotor rhythm.

3 METHODS

All methods are outlined in detail in the different papers. This is therefore a brief summary.

3.1 TRANSGENIC ANIMALS

Different transgenic zebrafish lines were used in order to target the different interneuron populations under investigation.

In paper 1, Tg[Chx10-GFP] was used to target the V2a interneurons for patch clamp recordings and for immunohistochemistry. Tg[GlyT2-GFP] was used for immunohistochemistry experiments as well as Tg[vGlut2a-LoxP-DsRed-LoxP-GFP] crossed with Tg[dbx1b:Cre] to verify possible dye coupling between motor neurons and glycinergic cells, and between motor neurons and V0v interneurons. In order to express halorhodopsin in motor neurons, the Gal4s1020t (Et(-0.6hsp70l:Gal4-VP16)s1020t) was crossed with Tg[UAS:NpHR-mCherry]s1989t, where Gal4-VP16 is expressed in motor neurons. The offspring were then crossed with Tg[Chx10-GFP] in order to enable patching from V2a interneurons while inhibiting motor neurons optogenetically. In paper 2, Tg[vGlut2a-LoxP-DsRed-LoxP-GFP] crossed with Tg[dbx1b:Cre] was used to target the V0v interneurons for patch clamp recordings as well as immunohistochemical experiments. In paper 3, Tg[GlyT2-LoxP-DsRed-LoxP-GFP] crossed with Tg[dbx1b:Cre] was used to target the V0d interneuron population for patch clamp recordings and immunohistochemistry.

3.2 ELECTROPHYSIOLOGY-ADULT ZEBRAFISH EX-VIVO PREPARATION

7-week-old fish were used for these experiments. To record from spinal neurons in the adult zebrafish, the fish is anaesthetized in MS-222 and pinned down in a Sylgaard-covered dish and eviscerated. The dissection is carried out in frozen slush of ringer solution. The skull, skin, brain and most of the musculature are removed and the brainstem-spinal cord preparation is placed in a recording chamber continuously perfused with ringer solution. Fictive locomotion is elicited by electrical stimulation of the brainstem-spinal cord interface. The extracellular recording is made from a motor nerve in the intermyotomal cleft of some muscle segments that are left intact at the caudal end of the preparation. Intracellular recordings are made from GFP-labeled neurons along a stretch of five segments in the mid-body region where bones have been removed to allow for access with the patch electrodes (Fig. 3).

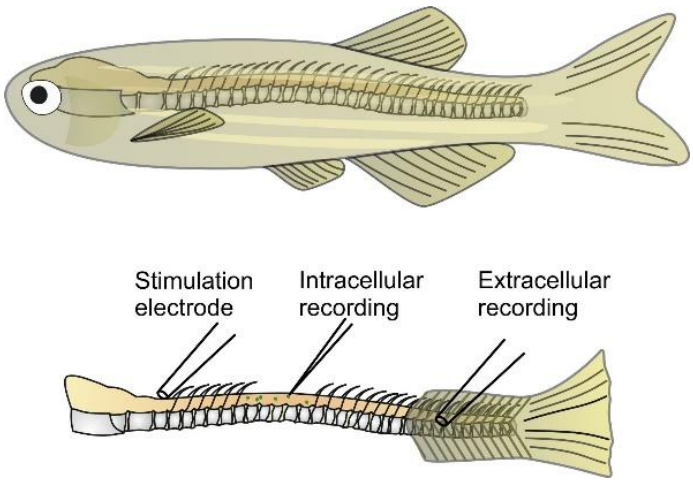

Figure 3. Cartoon of the adult zebrafish *ex-vivo* brainstem-spinal cord preparation. The brainstem and spinal cord are dissected out. Fictive locomotion is evoked by electrical stimulation at the brainstem-spinal cord interface (stimulation electrode). Motor nerve activity is recorded from the tail (extracellular recording). Single cell patch clamp recordings are performed in the mid-body region (intracellular recording).

3.3 ELECTROPHYSIOLOGY- LARVAL ZEBRAFISH PREPARATION

5-6-day-old larva are anaesthetized in MS-222 and pinned down in a Sylgard-covered recording chamber. The skin is removed and 6.25 μM α-bungarotoxin applied for approximately ten minutes until the neuromuscular junctions are blocked. The larva is subsequently dissected in normal ringer solution. The muscle fibers are removed from one or two segments in the mid-body region using finely etched tungsten pins to expose segments of spinal cord. Fictive locomotion is elicited by electrical stimulation of the otic vesicle, or the skin on the head. Intracellular recordings are made from GFP-labeled neurons in the spinal cord.

3.4 ANALYSIS OF NEURON SIZE AND POSITION

The dorso-ventral and rostro-caudal positions of the patch-clamped neurons were analyzed by measuring the distances from the neurons to the Mauthner axon and the dorsal- and lateral edges. Soma size was also measured. All measurements were carried out in ImageJ.

3.5 OPTOGENETICS

3-4-week-old zebrafish were used for optogenetic experiments. Halorhodopsin (NpHR) was expressed in motor neurons as described above. The motor neurons were inhibited over two spinal segments by shining 580 nm light via a custom-made LED system. To prevent the activation of GABAergic Kolmer-Agduhr cells, which also expressed NpHR in the animals used, GABAA receptors were blocked with gabazine.

3.6 2-PHOTON ABLATIONS

To study the contribution of the V0d interneurons to the locomotor pattern, 20-40 neurons were ablated unilaterally over five segments in the mid-body region of the 7-week-old brainstem-spinal cord ex vivo preparation. Ablations were carried out using a 2-photon laser (910 nm) and images before and after bleaching were captured to ensure the neurons were successfully ablated. The preparation was then flipped and extracellular recordings were performed along with intracellular recordings from slow motor neurons pre-labeled with rhodamine dextran.

3.7 IMMUNOHISTOCHEMISTRY

3.7.1 Neurobiotin stainings

0.25% neurobiotin was included in the patch pipette during intracellular recordings in order to obtain the morphology of the neurons. The preparations were treated according to staining protocol and the images of the neurons were obtained with a confocal microscope.

3.7.2 Anti-GFP and anti-glycine stainings

Antibody against GFP was used to enhance the signal of the labeled neurons to get an estimation of the number of interneurons per hemi-segment. An antibody against glycine was used to verify that V0d interneurons are glycinergic. All images were obtained using confocal microscopy.

3.7.3 Dye-coupling between motor neurons and interneurons

25% neurobiotin was injected into the musculature for retrograde uptake into the motor neurons and potential dye-coupled pre-motor interneurons. Fish were allowed to recover over-night and then processed according to staining protocol. Images of the presence or absence of dye-coupling were obtained by confocal microscopy.

3.8 BACKFILLING OF MOTOR NEURONS

Motor neurons were labeled for intracellular recordings by backfilling with rhodamine dextran 3000mw. A finely etched tungsten pin was covered in dye and carefully poked into the musculature of the fish in order to break motor neuron axons and allow for retrograde transport of the dye. Fish were allowed to recover for an hour minimum and subsequently dissected for patch clamp experiments.

4 RESULTS AND DISCUSSION

To address whether spinal interneurons and motor neurons are more diverse in their organization and function than previously thought, three projects were undertaken which make up this thesis.

4.1 PAPER 1: MOTOR NEURONS CONTROL LOCOMOTOR CIRCUIT FUNCTION RETROGRADELY VIA GAP JUNCTIONS

Traditionally, motor neurons have not been considered a part of the core CPG network in vertebrates. They were regarded merely as conveyers of the output signal to the muscle fibers rather than active entities partaking in the generation of the rhythm (Liddell and Sherrington, 1925; Sherrington, 1925; Grillner, S, Wallén, 1985; Duchateau J, 2011; Rybak, Dougherty and Shevtsova, 2015). In this paper, by looking at connections between motor neurons and the upstream V2a interneurons, we found that the motor neurons have an active, functional role in the generation of locomotion.

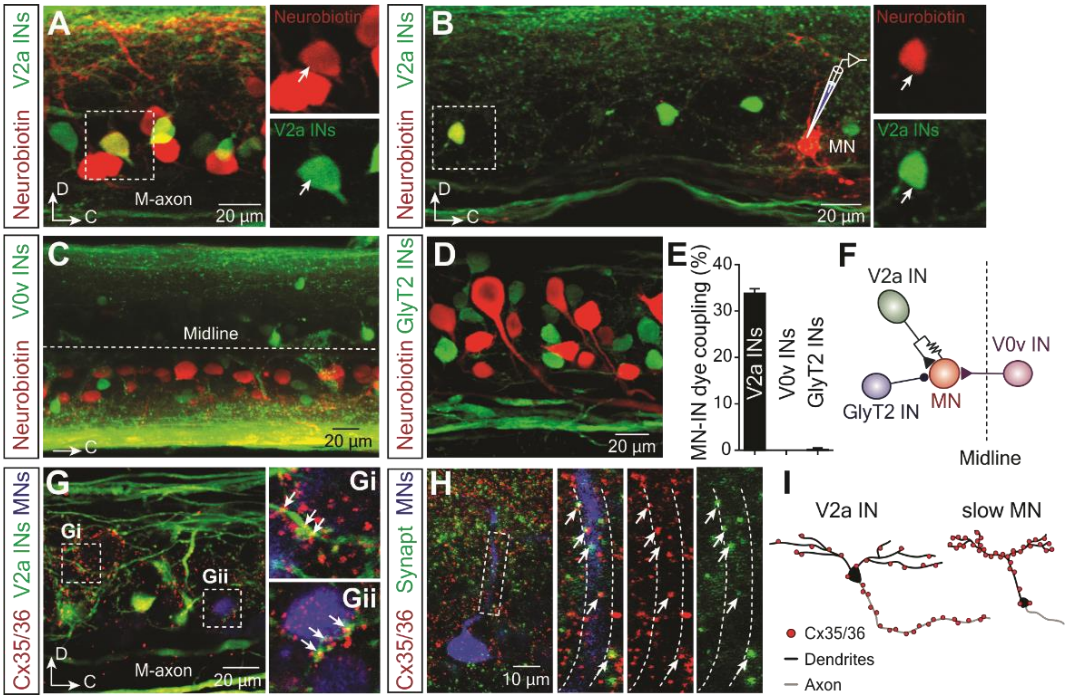

Figure 4. Dye-coupling indicates connections between motor neurons and V2a interneurons. (A-B) Dye-coupling occurred between motor neurons and V2a interneurons, but not motor neurons and V0v interneurons (C) or glycinergic interneurons (D). (E-F) Percentage of dye-coupling and diagram of connections from different interneurons onto motor neurons. (G-H) Stainings showing that V2a interneurons (green) and motor neurons (blue) are connected by connexin 35/36 (red) and that the pre-synaptic marker synaptophysin (green) is in close apposition with motor neurons (blue) and connexin 35/36 (red). (I) Cartoon showing where cx35/36 is densely located on V2a interneuron axons and slow motor neuron dendrites.

The V2a interneurons are ipsilateral, largely, albeit not exclusively, descending neurons which have been shown to be necessary and sufficient for the initiation of locomotion in the

zebrafish spinal cord (Eklof-Ljunggren *et al.*, 2012; Ljunggren *et al.*, 2014). Furthermore, these interneurons are connected to the motor neurons by both chemical synapses and electrical coupling via gap junctions (Ampatzis *et al.*, 2014). In this study, we showed that the V2a interneurons and the motor neurons display dye-coupling by injecting neurobiotin into the musculature of adult zebrafish and examining the spread of the dye between the motor neurons and the interneurons in the spinal cord. We concluded that gap junctions may be exclusively occurring between motor neurons and the V2a interneurons since no dye coupling was found between motor neurons and glycinergic neurons or motor neurons and commissural V0v interneurons. Staining for connexin 35/36 showed that gap junctions were located between V2a axons and motor neuron dendrites. Paired recordings from one motor neuron and one V2a interneuron showed that all neurons connected by gap junctions were also chemically connected.

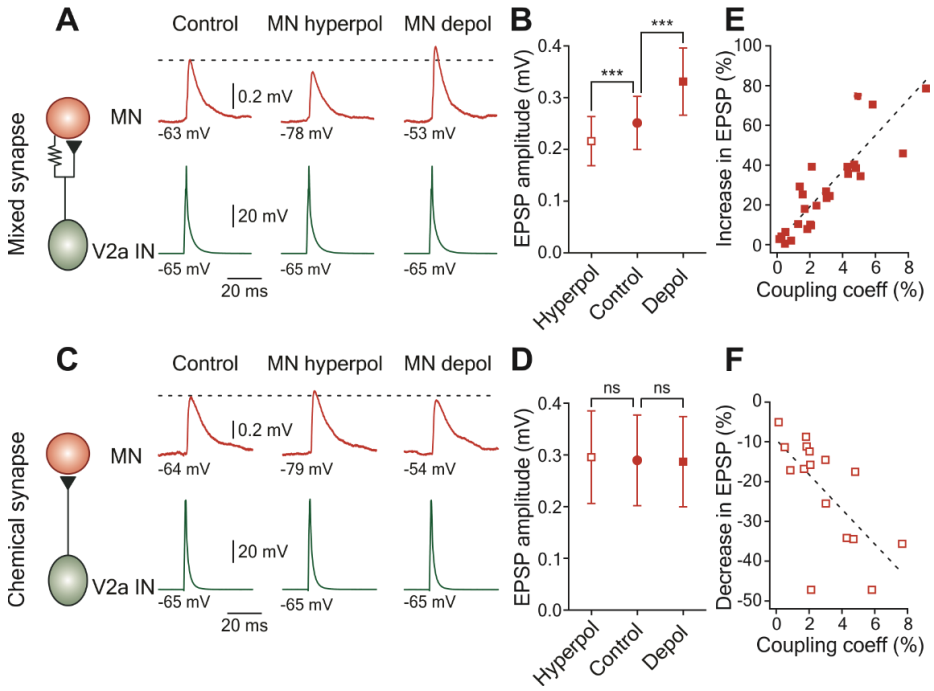

Figure 5. The motor neurons influence the strength of synaptic transmission. (A-B) MN membrane potential influences the EPSP amplitude in V2a-MN pairs connected by mixed chemical and electrical synapses. (C-D) MN membrane potential has no effect on the EPSP amplitude in V2a-MN pairs connected by chemical synapses only. (E) Increase in the coupling coefficient with the increase in EPSP amplitude for V2a-MN pairs with mixed synapses. (F) Decrease in the coupling coefficient with the decrease in EPSP amplitude for V2a-MN pairs with only chemical synapses.

Moreover, we showed that the membrane potential of the motor neurons could influence the firing threshold of the V2a interneurons during an injected current step. In addition, hyperpolarization of the motor neuron by injection of negative current during a swimming episode was sufficient to alter the recruitment pattern of the electrically connected V2a interneuron, but not a V2a interneuron only connected to the motor neuron by chemical synapses. These results suggest that the motor neurons can have a back-propagating influence on the locomotor rhythm by affecting the rhythm-generating V2a interneurons. In order to assess the acute effect of motor neuron de-recruitment on locomotion, we expressed halorhodopsin (NpHR) in the motor neurons and inhibited them during a locomotor episode

while performing patch-clamp recordings from GFP-labeled V2a interneurons. Inhibiting the motor neurons in two spinal segments lead to a significant decrease in swim frequency and swim bout duration. Furthermore, in some cases it de-recruited the V2a interneurons during swimming.

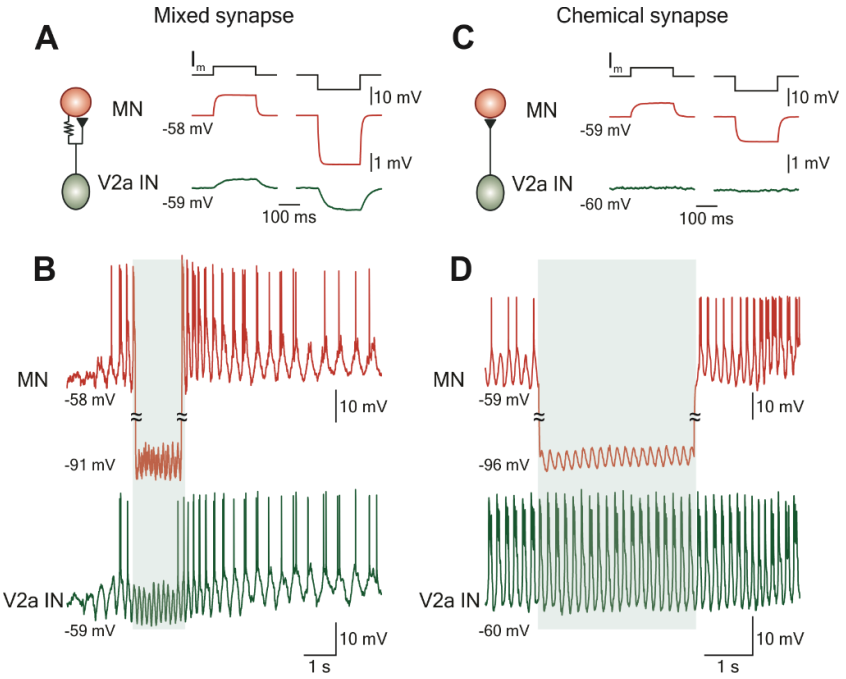

Figure 6. The motor neuron membrane potential influences V2a interneuron recruitment. (A) V2a-MN pair with mixed synapses. (B) Hyperpolarizing the MN during an episode of locomotion decreases the firing reliability of the V2a interneuron in a V2a-MN pair with mixed synapses. (C) V2a-MN pair with chemical synapses only. (D) Hyperpolarizing the MN during an episode of locomotion has no effect on the firing-reliability of the V2a interneuron in V2a-MN pairs with chemical synapses only.

In summary, we showed in this paper that the spinal motor neurons in zebrafish play an active role during locomotion, affecting V2a interneuron output via back-propagating electrical signaling. This finding indicates that the long-established view of the motor neurons as passive conveyers of upstream generated signals is incorrect, and that they instead are integrated components of the CPG network.

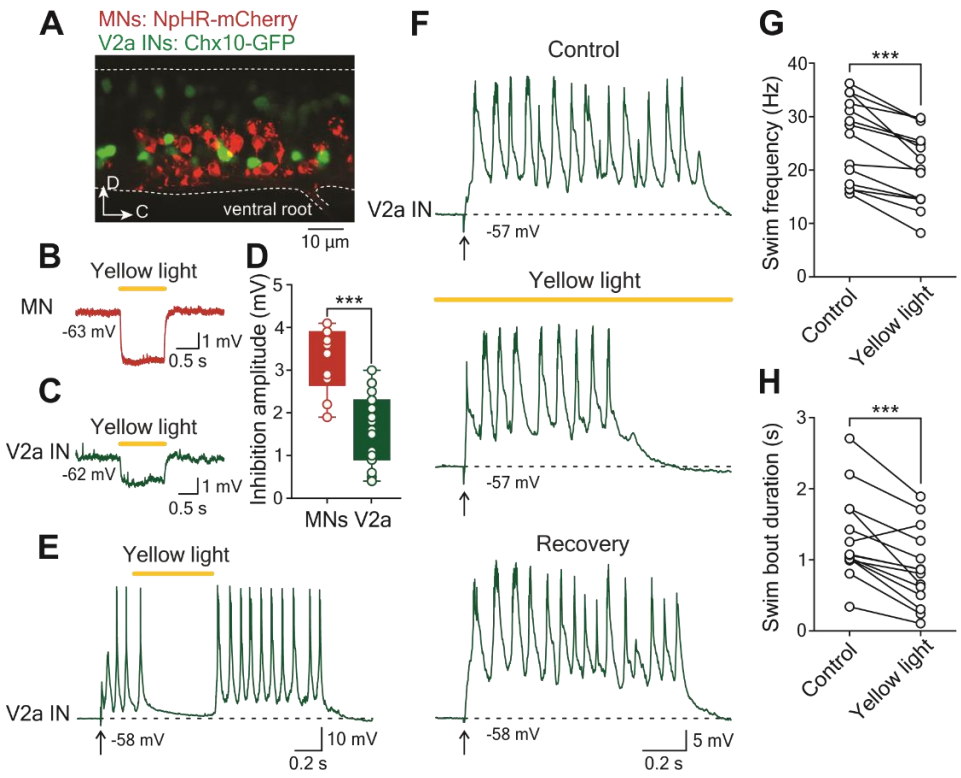

Figure 7. Optogenetic hyperpolarization of the motor neurons influences the locomotor frequency. (A) Halorhodopsin-mCherry was expressed in motor neurons and GFP in V2a interneurons. (B-D) optogenetic hyperpolarization with yellow light had a larger impact on the motor neurons than the V2a interneurons. (E) Hyperpolarization by yellow light reversibly stopped ongoing activity in V2a interneurons. (F) Hyperpolarization by yellow light had a reversible impact on swim frequency (G) and swim bout duration (H).

4.2 PAPER 2: FUNCTIONAL DIVERSITY OF EXCITATORY COMMISSURAL INTERNEURONS IN ADULT ZEBRAFISH

An important feature of locomotion is the ability to alternate the activity between different muscle groups, such as left-right alternation of axial muscle, which propels an animal like the fish forward. The neurons responsible for this are commissural neurons, which send their axons across the midline to innervate the contralateral side of the cord. One such population is the V0 interneurons, which arise from the p0 domain and can largely be divided into V0 ventral (V0v) and V0 dorsal (V0d) populations (Pierani *et al.*, 2001; Lanuza *et al.*, 2004; Goulding, 2009). The V0d population consists of inhibitory glycinergic/GABAergic neurons, whereas the V0v population is excitatory glutamatergic.

In the mouse, deletion of the V0 interneurons leads to a hopping gait (Lanuza *et al.*, 2004). Moreover, deletion of the V0d population leads to hopping at slow frequencies of locomotion, whereas deletion of the V0v population leads to hopping only at high frequencies (Lanuza *et al.*, 2004; Talpalar *et al.*, 2013). These results suggest that the V0 population is responsible for left-right alternation, with the V0d interneurons contributing towards alternation at lower frequencies, and the V0v interneurons at higher frequencies.

This experimental approach has treated the V0v and the V0d interneurons as homogenous entities, assuming that all the interneurons from the same population have similar properties and therefore carry out the same function in the network. A study of the V0 population in the larval zebrafish has shown that the V0v interneurons are very diverse in their morphology with the population including ascending, descending and bifurcating neurons. In addition, these neurons appear in the spinal cord in a specific order, with the ascending being born first, followed by the bifurcating and descending (Satou, Kimura and Higashijima, 2012). This study suggests that there is more diversity within the populations than has previously been assumed. Furthermore, single cell recordings from one larval subtype of V0v interneuron, the MCoD, have shown that they are active at low frequencies of locomotion and are actively inhibited when the rhythm speeds up (McLean *et al.*, 2007, 2008). The inconsistencies between the results obtained from mice and the larval zebrafish prompted us to probe the V0v population further, in the mature nervous system of the adult zebrafish.

This paper sought to determine whether the V0v interneurons in a mature CPG network have the same properties as one another and thus function as one unit, or whether they display a greater diversity as has been suggested from the anatomical studies in larval zebrafish.

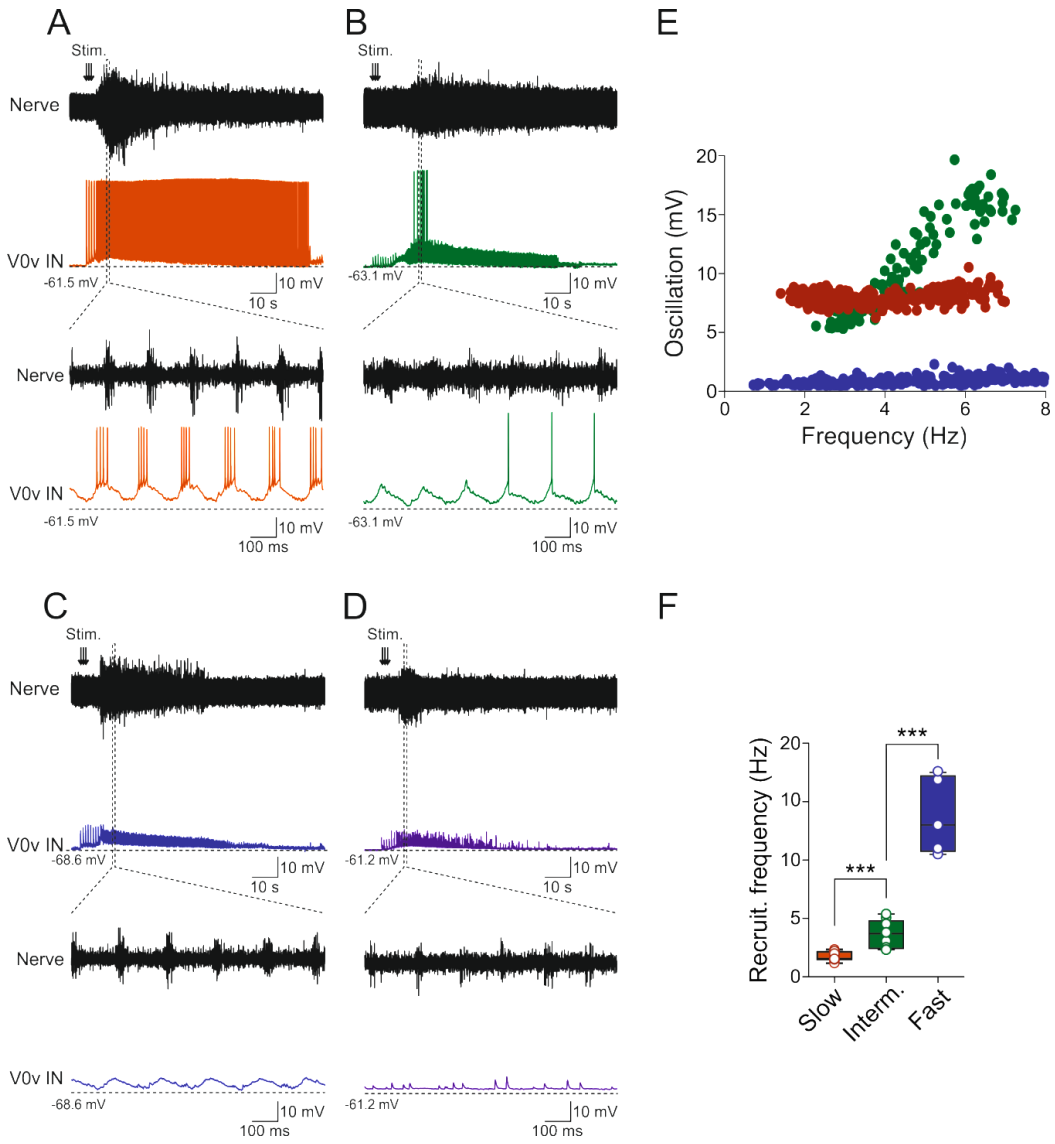

Figure 8. V0v interneurons comprise four different groups differentially active during locomotion. (A) Example of a slow V0v interneuron. These neurons fire action potentials on every locomotor cycle at all frequencies. (B) Example of an intermediate V0v interneuron. These neurons fire action potentials during a span of intermediate frequencies. (C) Example of a fast V0v interneuron. These neurons fire action potentials only during the fastest frequencies of locomotion. (D) Example of a non-patterned V0v interneuron. These neurons do not fire action potentials, nor do they receive any subthreshold oscillatory input. (E) Amplitude of the subthreshold membrane potential oscillations over swimming frequency for a slow (red), intermediate (green) and fast (blue) V0v interneuron. The slow V0v interneuron has even amplitude, indicating that it has reached its recruitment threshold. The intermediate V0v has increasing amplitude, suggesting it receives more excitatory input with higher frequency reaching towards recruitment threshold. The fast has a low, slowly increasing amplitude, suggesting it is far away from recruitment threshold. (F) Recruitment frequency for slow, intermediate and fast V0v. Data for the fast V0v interneurons is extrapolated. Data are mean ± SEM, P<0.0001.

We carried out single cell patch clamp recordings from GFP labeled V0v interneurons in the ex-vivo brainstem-spinal cord preparation and looked at the activity pattern of the neurons during electrically elicited fictive locomotion. We were able to identify four different types of locomotor-related activity patterns. The first we define as slow V0v interneurons, consisting of neurons that become active at the lowest frequencies of locomotion and remain active across the elicited frequency span. The second group is called intermediate, comprising interneurons that become active at an intermediate frequency span and remain active at frequencies above this. The third group we call fast, with interneurons being active only at the fastest locomotor frequencies, which were rarely obtained in our recordings. The fourth group we call non-patterned because these neurons did not appear to receive any rhythmic input during locomotion. The slow, intermediate and fast types had been previously identified for the motor neurons and the V2a interneurons, indicating that the different CPG neurons in the spinal cord all adhere to the same basic organization (Gabriel *et al.*, 2011; Ausborn, Mahmood and El Manira, 2012; Ampatzis *et al.*, 2013, 2014). The fourth, non-patterned group had not been identified before in the other neuron classes and thus appeared to be specific for the V0v class of interneurons. Hence, results from the single cell recordings indicated that the V0v interneurons are diverse in their activity pattern and do not contribute in the same way to the locomotor output.

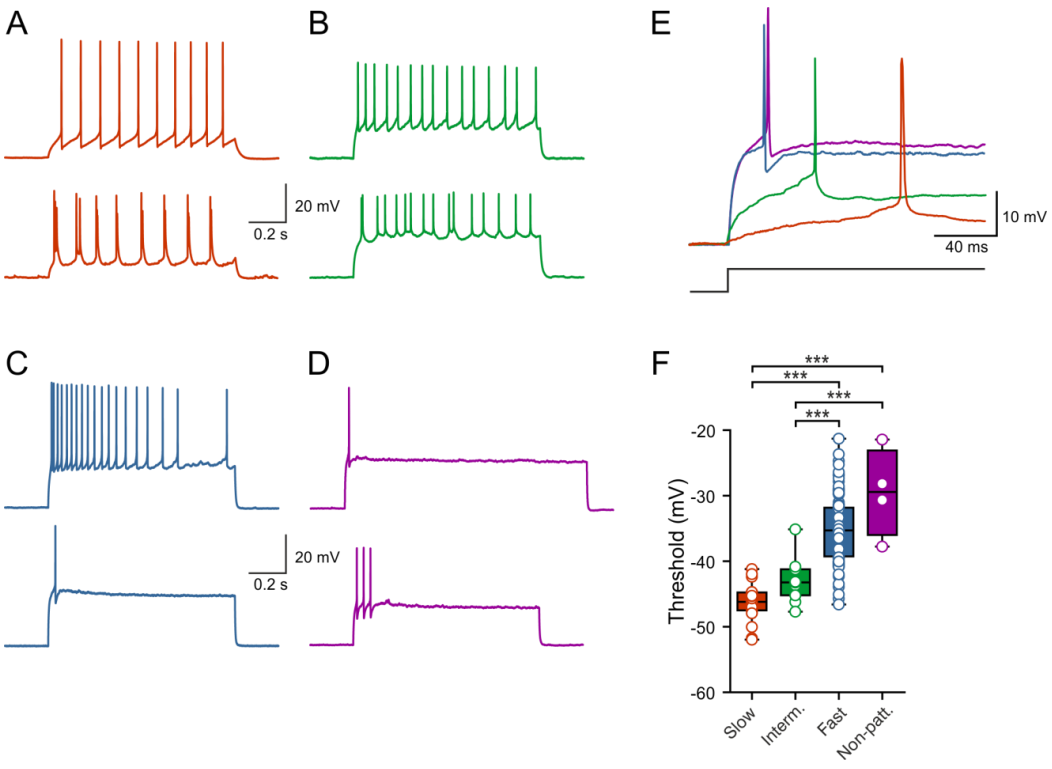

Figure 9. Intrinsic properties of the V0v interneurons. (A) Examples of two different slow V0v interneurons with a tonic versus a bursting firing pattern. (B) Examples of two different intermediate V0v interneurons with a tonic versus a bursting firing pattern. (C) Examples of two different fast V0v interneurons with a tonic-like and an adaptive firing pattern. (D) Examples of two different non-patterned V0v interneurons with firing properties with more or less adaptation. (E-F) Action potential threshold for slow, intermediate, fast and non-patterned V0v interneurons. Data are mean ± SEM, P<0.0001.

Next, we wanted to define the intrinsic properties of neurons from the four different groups, as well as the excitatory and inhibitory input they receive from the network during locomotion. We found that action potential threshold was different for the four different groups, with the slow having the lowest threshold followed by the intermediate, and the fast and non-patterned having the highest threshold. Inhibitory and excitatory inputs seemed to be quite uniform across the three groups that displayed rhythmic oscillations during locomotion. We therefore asked how the slow intermediate and fast interneurons could be recruited at such different frequencies. When we took into account the input resistance of the neurons and calculated the estimated received excitation we found that the fast V0v interneurons received less excitation than the slow and intermediate. Hence, it seems that the differences in the recruitment thresholds are the result of a combination of the input received and the intrinsic properties of the neurons themselves.

Furthermore, the different groups of neurons could fire action potentials in different patterns in response to a depolarizing current injection. The slow and intermediate V0v interneurons fired either in bursts or tonically, whereas the fast and non-patterned would fire with strong adaptive properties commonly eliciting only one action potential in response to the current injection. Interestingly, the morphology of the neurons, revealed by neurobiotin injections was variable just like previously described for the larval V0v interneurons (Satou, Kimura and Higashijima, 2012). However, the four different groups of V0v interneurons could not be discriminated based on morphological characteristics but rather had large intra-group variability.

Hence, with the data from this paper, we extended the knowledge about the V0v population of interneurons, showing that they display diversity in their properties, which in its turn indicates that they cannot be treated as one homogenous entity. Interestingly, in our study, a greater proportion of the neurons belonged to the fast group, suggesting that this population, despite its heterogeneity, may be primarily contributing towards alternation at fast locomotor frequencies. This is in accordance with the results from mice, since ablating the V0v interneurons in mice leads to a disruption of left-right alternation only at higher frequencies of locomotion (Lanuza *et al.*, 2004; Talpalar *et al.*, 2013).

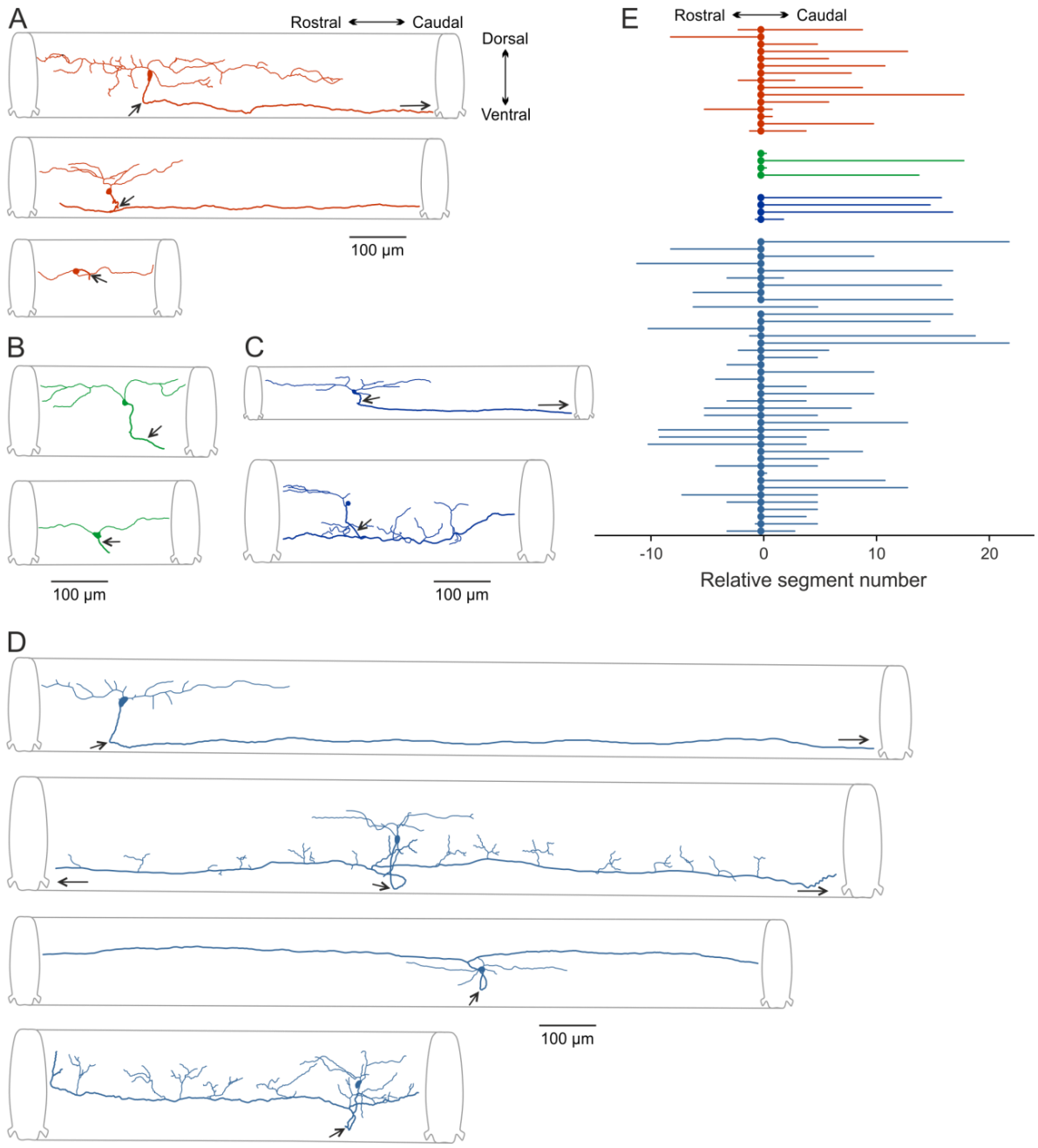

Figure 10. **V0v interneurons display large variation in their morphologies.** Neurobiotin injections during recordings revealed largely four different morphologies: descending, ascending, bifurcating and local. No specific morphology was exclusively found in only one of the functionally distinct groups of V0v interneurons. Representative morphologies of slow (A), intermediate (B), non-patterned (C) and fast (D) V0v interneurons. (E) Shows a diagram of the number of segments the axonal processes stretched in the ascending and the descending direction. Soma position is set to '0'.

4.3 PAPER 3: MATURATION OF INHIBITORY V0 INTERNEURON DIVERSITY IN THE ZEBRAFISH LOCOMOTOR NETWORK

In this study, which follows up on the previous paper, we investigated the inhibitory V0d interneurons. These interneurons have been found to be important for left-right alternation at low frequency locomotion in the mouse, yet little is known regarding this group of neurons in the zebrafish. Morphological studies in the larval zebrafish have shown that they consist of a glycinergic and a GABAergic population. The GABAergic neurons are local and can have axonal projections both ipsi- and contralaterally, whereas the glycinergic neurons are commissural, mainly bifurcating with one axonal process, either the descending or the ascending, extending further than the other (Satou, Kimura and Higashijima, 2012). Studies of bifurcating glycinergic interneurons in the larval have shown that these neurons are active during swimming mainly at higher frequencies (Liao and Fetcho, 2008; Kishore *et al.*, 2015).

In our study, we decided to confirm the previously reported activity pattern and morphology of the larval glycinergic V0d interneurons and investigate whether the findings would be maintained throughout development, such that we would find the same organization of the adult V0d interneurons.

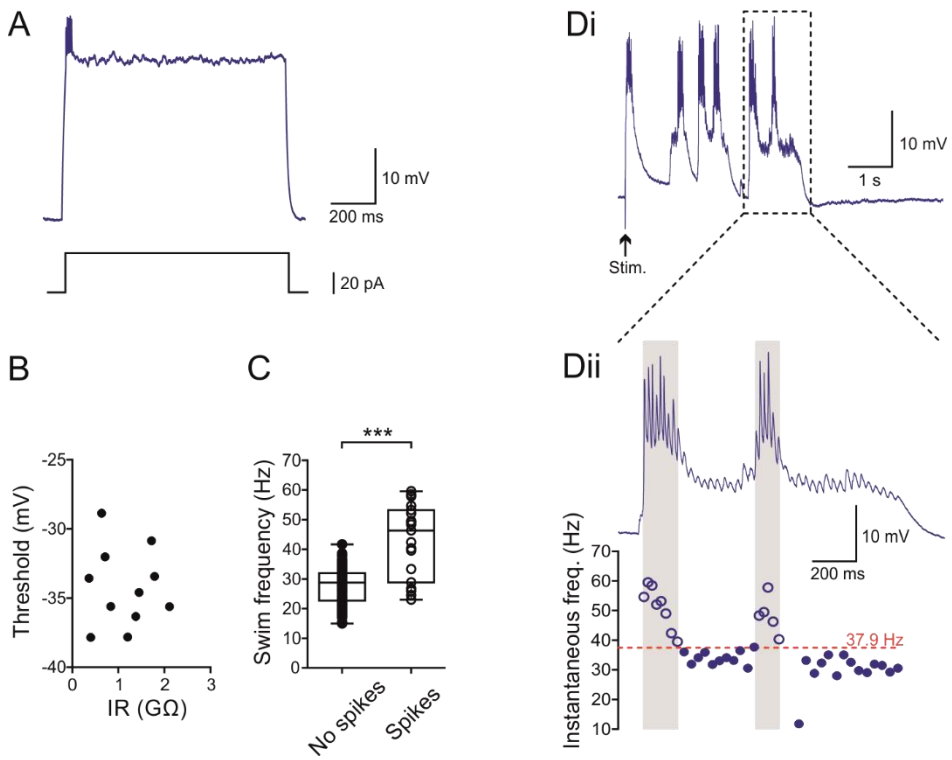

Figure 7. Larval glycinergic V0d interneurons are homogeneous in their properties. (A) Larval V0d interneurons display adaptive firing properties in response to a depolarizing current injection and high action potential threshold (B). (C) Locomotor cycles with action potentials were more frequent at higher frequencies of swimming. Data are mean ± SEM, P<0.0001. (Di) Example of activity from a larval V0d interneuron during swimming episodes. (Dii) Expansion of one episode from Di showing recruitment of the neuron when the instantaneous frequency reaches above 37.9 Hz.

First, we performed patch-clamp recordings from V0d interneurons in the larval zebrafish. We found that the larval V0d interneurons could indeed be described as a homogenous group of neurons, since they displayed little variation in firing pattern and morphology. They had relatively high thresholds for firing action potentials, had an adaptive firing pattern and all appeared to have bifurcating morphologies. Moreover, they tended to be recruited only during high frequency swimming. These results, along with previously reported observations, suggest that the larval glycinergic V0d interneurons are all involved in mediating a similar function in the developing CPG network, and are primarily involved in fast swimming.

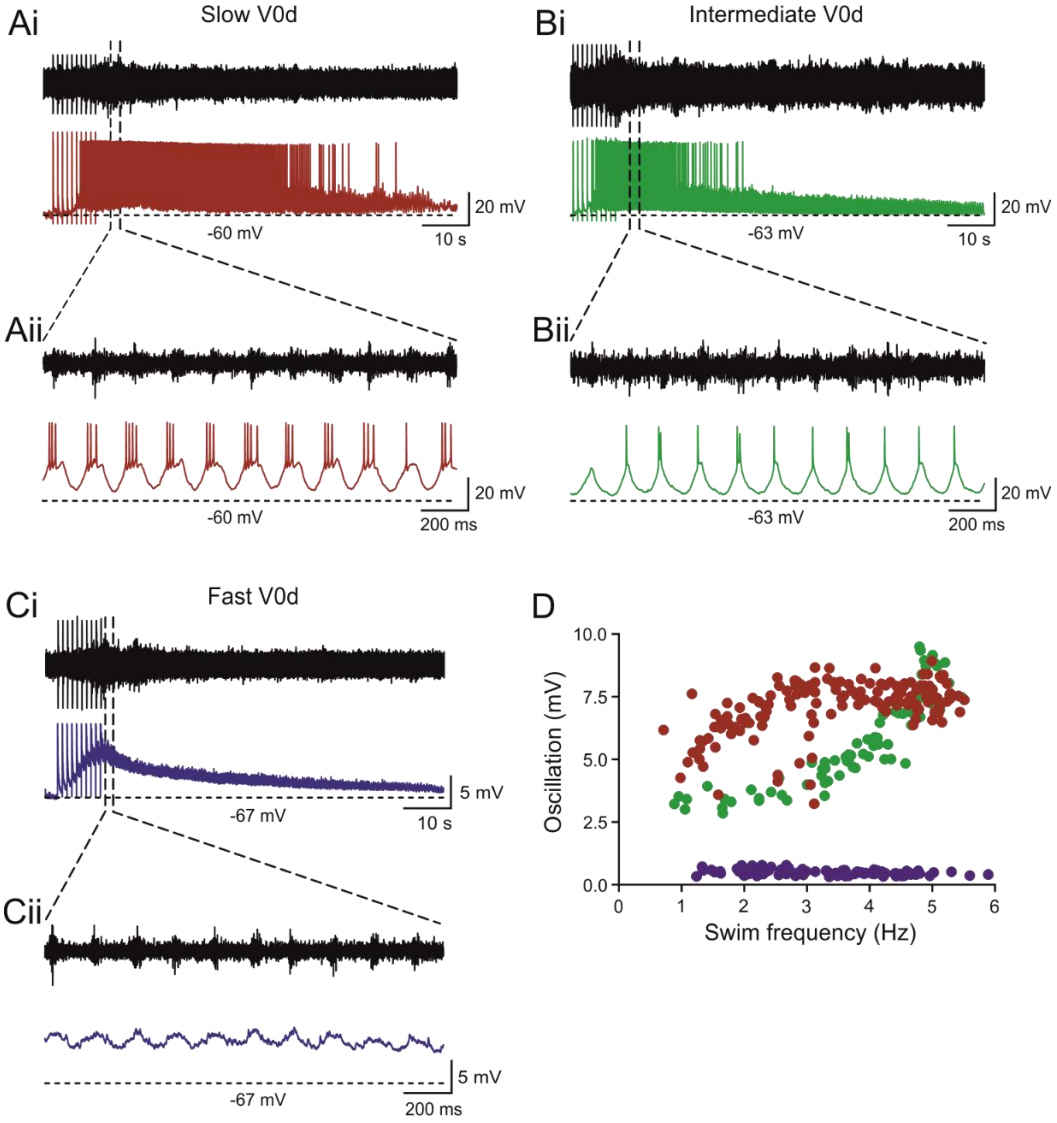

Figure 8. Adult V0d interneurons comprise three different groups differentially active during locomotion. (Ai-Aii) Slow V0d interneurons are recruited at the lowest frequencies of locomotion and remain recruited throughout the episode. (Bi-Bii) Intermediate V0d interneurons become recruited at intermediate frequencies and de-recruit when the frequency drops below their recruitment frequency. (Ci-Cii) Fast V0d interneurons are only recruited at fast frequencies of locomotion but receive sub-threshold oscillatory input. (D) The sub-threshold oscillation amplitude remains relatively constant for the slow V0d interneurons across the swimming frequencies (red), increases with swimming frequency for the intermediate (green) and increases very slowly for the fast V0d interneurons (blue).

Next, we sought to investigate the morphology and properties of the glycinergic V0d interneurons in the adult zebrafish. We first confirmed that the neurons were indeed

glycinergic and that they had commissural axons that cross the midline to innervate the opposite side of the spinal cord. Next, we examined their activity pattern during locomotion. The adult V0d interneurons did not exhibit the same homogeny as they did at the larval stage. Instead, interestingly they could be divided into the same groups that have been found in the motor neurons and the V2a and V0v interneurons: slow, intermediate and fast. In accordance with what we had previously found for other neuron classes in the adult spinal cord, the slow had the lowest threshold for firing action potentials, followed by the intermediate and the fast had the highest thresholds. The slow and the intermediate V0d interneurons could fire action potentials either tonically or in bursts of action potentials, whereas the fast could fire either tonically or with a strong degree of adaptation. Similarly to what we had found for the V0v population, the V0d interneurons displayed a large variability in their morphologies, however this variability was unrelated to the three functionally different groups. Interestingly, there were a greater proportion of slow V0d interneurons than intermediate or fast in our randomly collected dataset. This is in contrast with the overrepresentation of fast interneurons in the V0v population described in the preceding paper, and suggests that in the adult zebrafish the V0d interneurons may be primarily involved in slow swimming. Ablation experiments from mice have suggested that the V0v interneurons are responsible for left-right alternation at high frequencies and V0d interneurons at low frequencies (Talpalar *et al.*, 2013). Perhaps the overall result of deleting an entire population is a reflection of the function of the majority of the neurons in that population, and not necessarily the entire population as a whole.

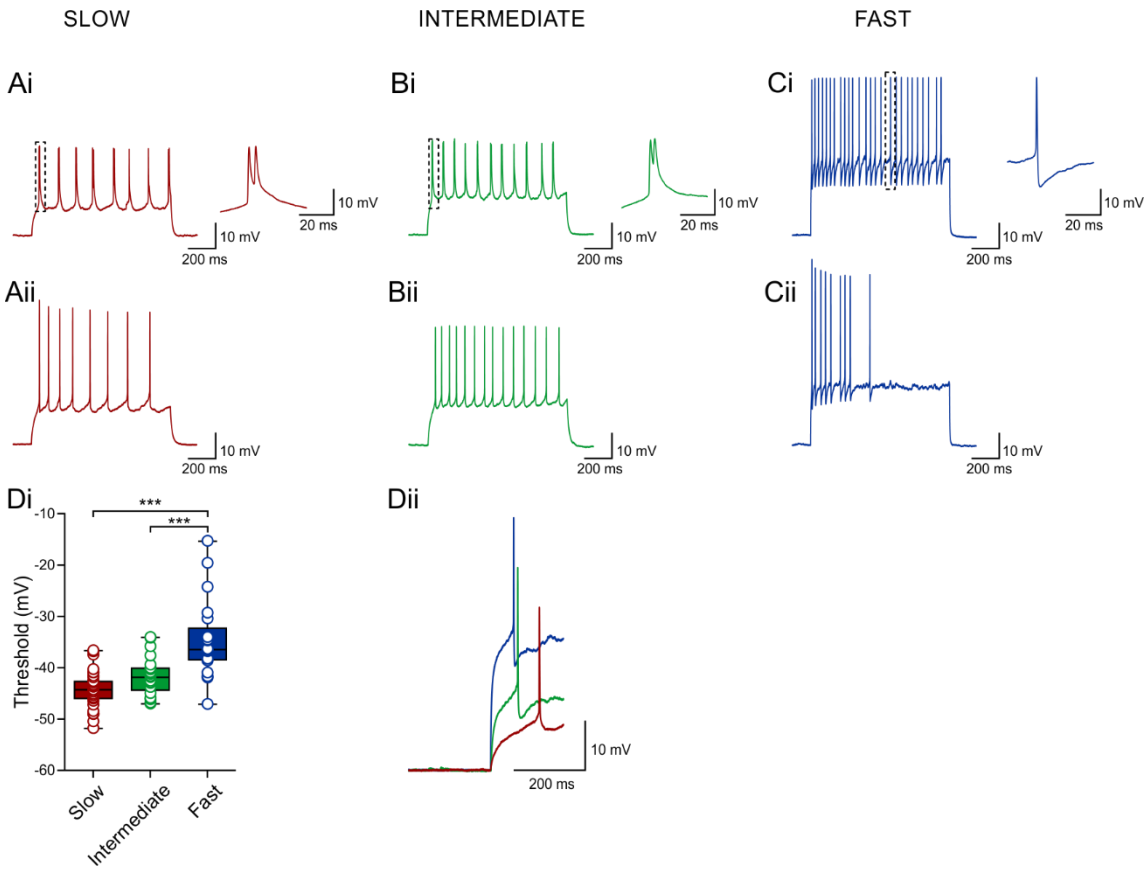

Figure 9. Intrinsic properties of the V0d interneurons. Examples of firing patterns from two different V0d interneurons from each group. (Ai-Aii) Slow V0d interneurons were either burst-firing or tonic, as was intermediate V0d interneurons (Bi-Bii). (Ci-Cii) Fast V0d interneurons were either tonic or had strong adaptive properties. (Di-Dii) Action potential threshold varied with the slow V0d interneurons having the lowerst threshold (red) followed by the intermediate (green) and fast (blue). Data are mean ± SEM, $P<0.0001$.

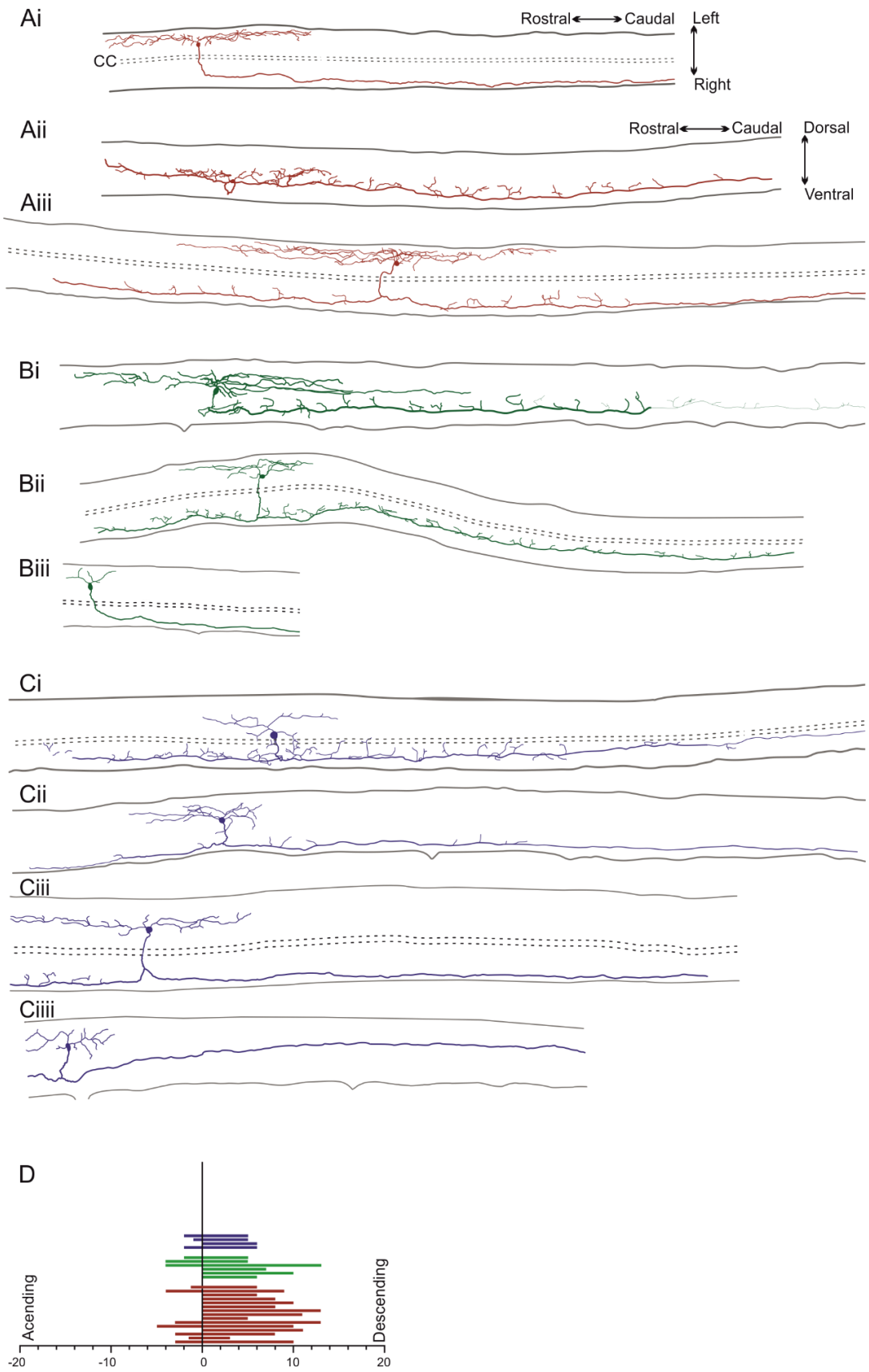

Figure 10. Adult V0d interneurons display large variability in morphological characteristics. V0d interneurons were bifurcating or descending and both morphologies were represented in all three groups. Representative morphologies for slow (Ai-Aiii), intermediate (Bi-Biii) and fast (Ci-Ciiii) V0d interneurons. (D) Shows a diagram of the number of segments the axonal processes stretched in the ascending and the descending directions. Soma position is set to '0'.

Taken together, our results from the experiments in the adult zebrafish show that the V0d interneurons undergo considerable developmental changes during the course of maturation into adulthood. The neurons establish three functionally different groups active at different frequency spans during locomotion, instead of all contributing equally to the locomotor rhythm. Furthermore, the great diversity in morphology in adulthood, compared to the relative homogeny at the larval stages, further indicates that dramatic changes take place as the system develops.

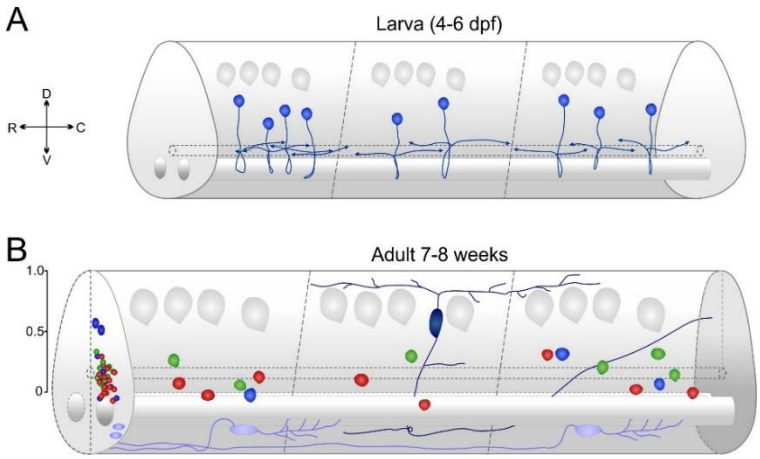

Figure 11. Schematic of the distribution of V0d interneurons at two stages of development. (A) Larval V0d interneurons are homogeneous in their morphology and properties. (B) Adult V0d interneurons can be active at slow (red), intermediate (green) or fast (blue) frequencies and are unevenly distributed in the spinal cord.

In addition to the aforementioned groups of neurons, we identified two types of neurons in the adult zebrafish spinal cord that were easily identifiable in terms of their position in the spinal cord, morphology, and firing pattern, allowing us to characterize these subtypes in detail. The first type occupied an extreme ventro-lateral position in the spinal cord and had a distinct morphology. It was the only V0d interneuron we found to have a purely ascending contralateral axon, stretching 9-11 segments rostral to the soma position. The soma had an oval shape and possessed thick dendrites in the rostral and caudal directions, with several dendritic processes extending in a dorso-medial direction, seemingly towards the Mauthner axon. There was on average one such neuron per hemi-segment, although they were not always evenly distributed along the length of the spinal cord. These neurons displayed adaptive firing properties, with a surprisingly low threshold for firing action potentials. During locomotion they behaved like the fast group of V0d interneurons, displaying rhythmic, low amplitude membrane potential oscillations, indicating that they likely play a role in faster swimming.

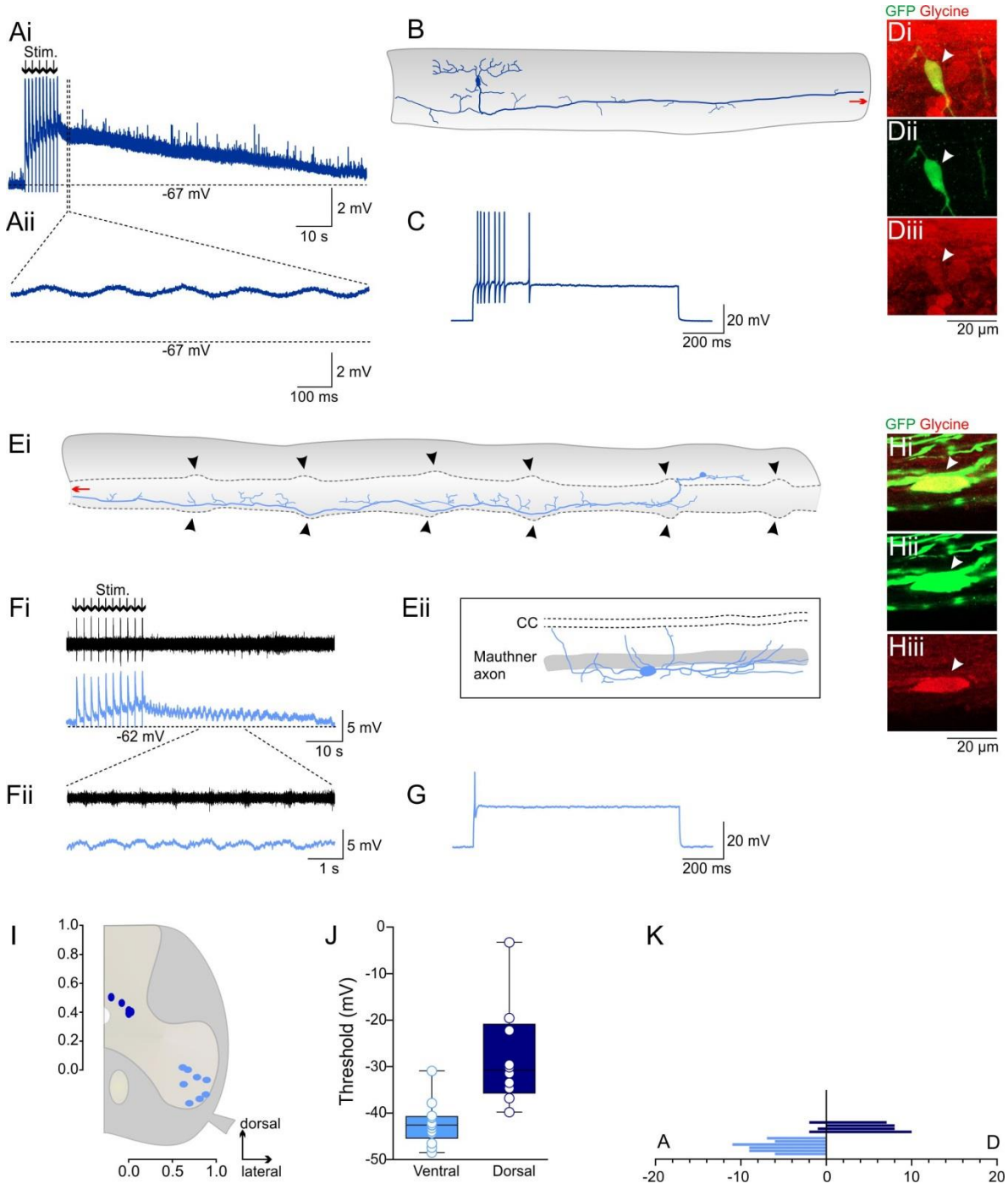

Figure 12. Characterization of two adult V0d interneuron types. (Ai-Aii) The first type has activity pattern reminiscent of the fast V0d interneurons. (B) This neuron has thick dendrites and an axon descending 8-10 segments (K). It displays adaptive firing properties with a high threshold (C, J) and occupies a dorso-medial position in the spinal cord (I). (Fi-Fii) The second type has an activity pattern in accordance with a fast V0d interneuron and adaptive firing properties with a low threshold (G, J). This neuron has an axon ascending for 6-11 segments (Ei, K) and thick dendrites stretching towards the mauthner axon (Eii). It occupies a latero-ventral position in the spinal cord (I). Both neurons are are glycinergic (Di-Diii and Hi-Hiii).

The second type of neuron we could specifically characterize occupied a dorso-medial position in the spinal cord, and was located in close proximity to the primary motor neurons. It had a large soma with one, thick apical dendrite splitting in two and heading in a rostral and caudal direction on the ipsilateral side of the cord. The axon was commissural and descended for 8-9 segments, with distinctive collaterals in close proximity to the primary motor neurons on the contralateral side. There were only approximately three of these neurons per hemi-cord. The threshold for firing action potentials was high, and the neurons displayed adaptive firing properties. During locomotion, they also displayed rhythmic, low amplitude membrane potential oscillations, characteristic of the fast neurons.

The inhibitory commissural interneurons have been shown, using approaches such as pharmacological blockage, hemi-cord transection and transcription factor-driven ablations, to be important for left-right alternation during locomotion across many different vertebrates (Buchanan, 1982, 1999; Cohen and Harris-Warrick, 1984; Dale, 1985; McPherson, Buchanan and Kasicki, 1994; Cowley and Schmidt, 1995; E. Kremer, 1997; Kjaerulff and Kiehn, 1997; Lanuza *et al.*, 2004; McDearmid, 2005; Talpalar *et al.*, 2013). Blocking inhibition using the glycine antagonist strychnine abolishes left-right coordination in the adult zebrafish ex-vivo preparation (Gabriel *et al.*, 2008; Kyriakatos *et al.*, 2011). Hence, we expected that a removal of the glycinergic V0d interneurons would have an effect on the swimming rhythm in the adult zebrafish. We used a two-photon laser to selectively ablate 20-40 V0d interneurons in one hemi-cord in the mid-body region of the ex-vivo brainstem-spinal cord preparation. After ablations, swimming could be elicited as normal, however the swimming episodes appeared disrupted and shorter than normal. When measuring the trough value of the mid-cycle inhibition we could see that the value became more depolarized over time, indicating that the inhibition is insufficient to carry out its proper function throughout the swim episode. Therefore, we concluded that, although not crucial for the locomotor rhythm, the V0d interneurons are important for appropriate maintenance of mid-cycle inhibition during swimming. In summary, the data from this paper showed that one interneuron class, the V0d interneurons, change their properties over the course of development such that their function becomes more varied as the network matures.

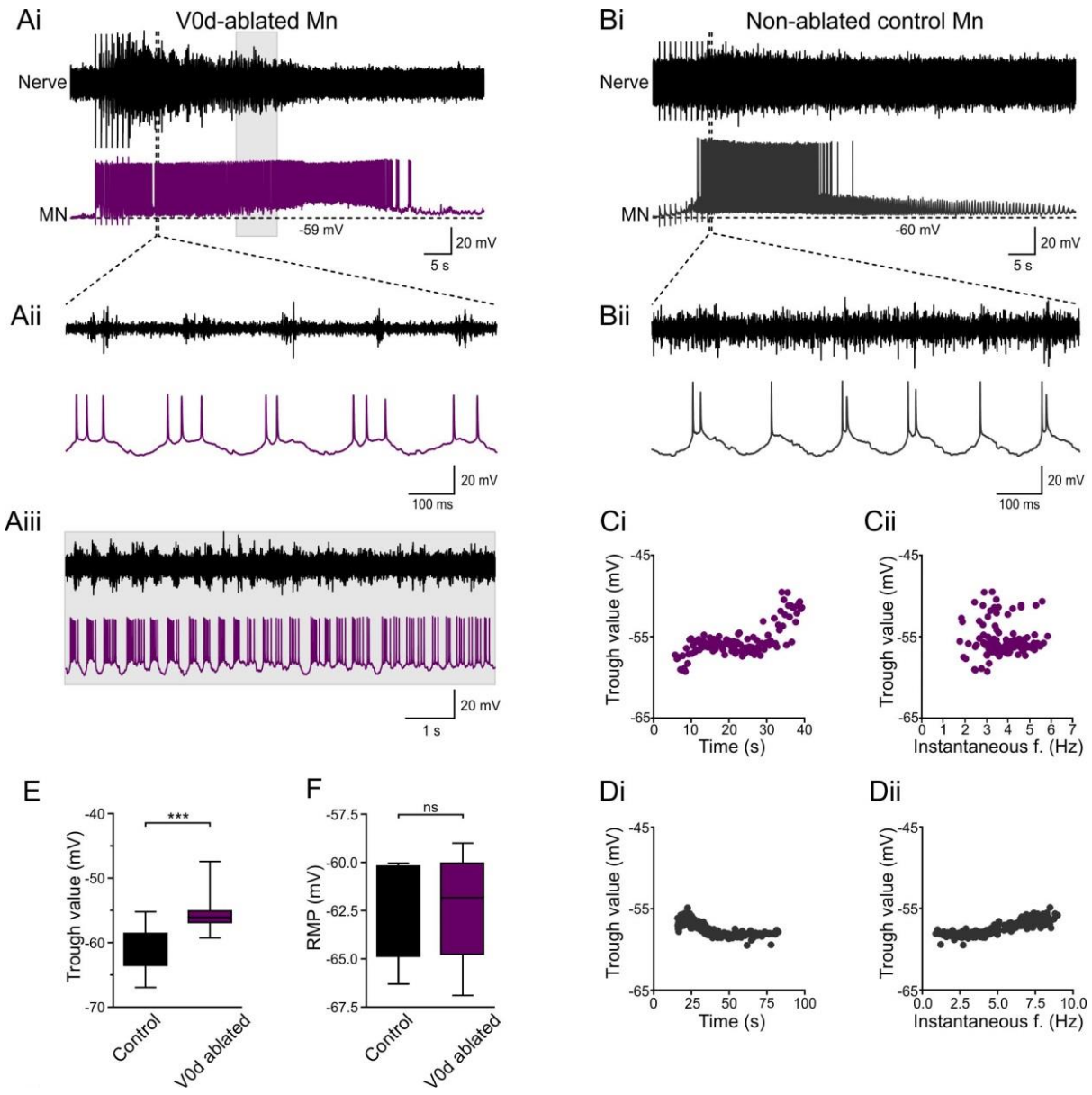

Figure 13. Ablation of V0d interneurons disrupts mid-cycle inhibition. (Ai-Aiii) Ablating 20-40 V0d interneurons affects the mid-cycle inhibition and disrupts the ongoing locomotor episode compared with control conditions (Bi-Bii). (Ci-Cii) trough amplitude is disrupted over time and appears scattered by comparison to control (Di-Dii). Mean trough value is significantly depolarized in ablated preparation compared to controls (E) data are mean ± SEM, $P<0.0001$. (F) There is no difference in resting membrane potential between motor neurons in ablated preparations and non-ablated controls.

5 CONCLUSION AND FUTURE PERSPECTIVES

The overall aim of this thesis was to shed light on the organization and possible functional contributions of different interneurons and the motor neurons in the spinal cord. The most recent approach to understanding CPG network organization has been to target interneurons and motor neurons that are genetically related to each other and treat them as whole, homogeneous populations contributing equally to the locomotor output. The work encompassing this thesis sought to probe these neuronal populations on a cellular level in order to determine if there is a greater diversity in their properties and functional contribution than has previously been thought. To this end, the zebrafish as a model system is amenable to genetic manipulations as well as detailed mapping of properties at the single cell level while keeping the locomotor network intact.

The work in this thesis shows that one neuron class in the spinal cord, the V0 interneurons, is more heterogeneous in its properties than what has been reported from experiments in the mouse. It shows that probing the function of populations of neurons at the network level based on their expression of transcription factors needs to be done with caution since not all neurons within the same class necessarily have the same properties and contribute in the same manner to the locomotor rhythm. Furthermore, it shows that one neuronal population undergoes developmental changes such that it adopts a new functional organization as the animal matures. This indicates a need for awareness when choosing the experimental model, the stage of development, and caution interpreting the results gained.

In addition, we have uncovered a previously undescribed contribution of the spinal motor neurons to the locomotor activity. They are not merely passive conveyers of the upstream generated locomotor programs, but can actively modify these programs by means of retrograde signaling directly to the rhythm-generating interneurons. Taken together, these results add another level of complexity to an already intricate circuit organization.

Although, considerable information can be obtained from single cell recordings, many questions remain regarding the functional contribution of the different neuron populations in the spinal cord. The V0 interneurons have been suggested to be responsible for left-right alternation (Lanuza *et al.*, 2004; Talpalar *et al.*, 2013). Although the work in this thesis starts to address the functional contribution of the V0d interneurons to locomotion by ablation studies, it would be valuable to perform similar experiments in the larval zebrafish. In addition, similar ablation experiments of the V0v interneurons in both larval and adult zebrafish would be beneficial to get a more complete understanding of the contribution of the V0 interneuron class as a whole. Although fictive swimming is a useful output for the analysis of changes in the locomotor pattern and has many advantages, it is not the equivalent of free swimming. Analysis of freely behaving animals, in which specific neuronal types have been ablated, could help understanding the behavioral consequences of the disruption of a specific neuronal class.

Although output in the shape of fictive locomotion or behavior can tell us why a specific group of neurons might be important, it will not explain how or by what mechanisms. In order to gain a full understanding of the locomotor CPGs we have to address the question of who is talking to whom in the network. Work in this thesis has demonstrated an unforeseen role for the otherwise well characterized motor neurons; a role which could only be properly elucidated by pairwise recordings from single identified neurons in the network. Such systematic paired recordings between neurons of the same population, and neurons of different populations, would help us explain by what means a specific neuron is carrying out its function in the circuitry. A thorough and detailed understanding of how the locomotor circuitry is built and how it operates on every level is necessary if we want to understand the underlying cause of impairments to the locomotor system, manifested by symptoms seen in diseases such as Parkinson's disease, Huntington's disease and many more. We cannot heal a broken system if we do not have the knowledge of how that system is built and organized on a detailed level.

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Ok, I admit, sentimental stuff is not my thing. However, six years is a long time to spend dissecting fish, so there have been a few people around during this time.

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